University of Alberta

The Influence of Recombinant Bovine Growth Hormone and Growth Hormone Releasing Factor on Fat Synthesis in Primiparous Holstein Cows.

By

Naomi Simone Beswick ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Animal Science

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall 1997

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Influence of Recombinant Bovine Growth Hormone and Growth Hormone Releasing Factor on Fat Synthesis in Primiparous Holstein Cows by Naomi Simone Beswick in partial fulfillment of the requirements for the degree of . Master of Science in Animal Science.

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Abstract

Thirty primiparous Holsteins were received either recombinant bovine growth hormone (bGH), growth hormone releasing factor (bGRF), or no treatment. Both treatments elevated milk production and nonesterified fatty acid concentration without changing milk fat percentage. We examined acetyl-CoA carboxylase and fatty acid synthase as a measure of de novo synthesis, and lipoprotein lipase as an indicator of fat uptake in the mammary gland and omental fat. Finally, stearoyl-CoA desaturase was examined as an indication of fatty acid desaturation. Both bGH and bGRF treatment were hypothesized to result in elevated lipid metabolism in the mammary gland and reduced lipid metabolism in adipose tissue. Neither treatment exerted a significant effect on the parameters measured in the mammary gland, while both treatments significantly reduced all the omental fat measurements. We conclude that the majority of substrate required for increased milk fat synthesis may be provided by the increased uptake of nonesterified fatty acids.

Acknowledgements

I would especially like to thank my supervisory committee, John Kennelly, Wally Dixon and Erasmus Okine for their input and advice throughout this process. I would also like to thank Renate Meuser for all of her technical assistance and encouragement. I am also grateful to other graduate students in our research group and laboratory who always made this process a pleasant one. Finally, I would like to acknowledge my husband, Gary Nugent, and my family for always supporting me.

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List of Abbreviations

ACC	acetyl-CoA carboxylase
ACP	acyl carrier protein
bGH	recombinant bovine growth hormone
bGRF	bovine growth hormone-releasing factor
FAS	fatty acid synthase
GH	growth hormone
GRF	growth hormone-releasing factor
HSL	hormone sensitive lipase
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
LCFA	long chain fatty acids
LpL	lipoprotein lipase
MCFA	medium chain fatty acids
mRNA	messenger RNA
NEFA	non-esterified fatty acids
SCD	stearoyl-CoA desaturase
SCFA	short chain fatty acids
SS	somatostatin
ST	somatotropin
VLDL	very low density lipoproteins

Chapter 1 - Introduction

The Role of Bovine Growth Hormone and Growth Hormone-Releasing Factor in Fat Synthesis of the Holstein Cow.

Introduction

Lactation is a complex physiological process that is mediated by hormones which influence mammary gland development and redirect nutrients to the mammary gland from other tissues. One of the key lactogenic hormones in ruminants is growth hormone (GH). Administration of the recombinant and pituitary derived versions of this hormone increases yield of milk and milk components (5, 8, 20, 21, 22, 27, 28, 44, 49, 68, 71, 76). The composition of the milk of animals treated with this hormone is generally unaltered, although there have been reports of increases in milk fat percentage (10, 45). In order to understand the exact means by which growth hormone stimulates milk production, and in particular its impact on milk fat synthesis, we designed an experiment in which we examined the influence of bGH and bGRF on enzymes involved in fat metabolism. This chapter provides background information on the control of initiation and maintenance of lactation with particular reference to the hormones and enzymes evaluated in this study.

1.1 - Hormones

The onset of lactation in the bovine is mediated by a hormonal cascade that begins prior to parturition. In the latter portion of gestation, progesterone begins to decline and drops off dramatically at parturition (30). During gestation when the progesterone concentration is high, the synthesis of lactose is inhibited thus preventing commencement of milk production. At this time estrogen and placental lactogen concentrations are high (5). Elevated estrogen stimulates the release of prolactin from the anterior pituitary which then acts on the mammary gland to maintain the functional and structural integrity of the cells, and to stimulate milk production as progesterone levels decline. Placental lactogen is similar in structure to GH and prolactin and acts upon adipose tissue, mammary gland and liver. At the end of gestation, when estrogen levels are elevated and progesterone levels are depressed, mammary development and lactose synthesis are stimulated. Oxytocin is also secreted from the posterior pituitary which stimulates uterine contractions and milk secretion. At this stage GH, which is accepted as the principle hormone involved in the maintenance of milk production, (29) is also elevated. Growth hormone acts directly on tissues such as adipose tissue and liver, and indirectly on the mammary gland, although there is some evidence to suggest that the action on the mammary gland may also be direct. This results in nutrient partitioning to the mammary gland, and the synthesis of insulin-like growth factor I (IGF-I) in the liver, which acts on the mammary gland and is involved in the process of nutrient partitioning. The hormonal changes occur in concert to initiate lactation.

1.2 - Lactation Curve

Lactation in the bovine is comprised of three distinct periods: early, mid and late lactation, and lasts approximately 305 d followed by a 60 d dry period. Milk yield follows a defined pattern which can be used to create a lactation curve (Figure 1.1). Early lactation is defined as the first twelve weeks of lactation (79). During this time, the animal reaches peak production. After parturition, milk production gradually increases reaching a peak at 4 to 8 weeks post-partum. Administration of bGH helps to maintain the peak and persistency of the lactation curve. The exact time at which the animal's milk production peaks can vary, and is a reflection of the nutritional and health status of the animal. In early lactation, the animal is in negative energy balance as milk yield peaks prior to the peak in feed intake. Adipose tissue is mobilized to provide the additional substrate needed to meet the energy requirements for milk production. In addition, lipogenesis in adipose tissue is suppressed in order to favour milk fat production. Adipose tissue mobilization acts as a source for long chain fatty acids which can be incorporated into milk fat. During early lactation, the milk fat percentage tends to be lower than in mid or late lactation. In addition, during early lactation C6 - C16 tend to be higher in concentration and C4 and C18:0 - 18:2 tend to be reduced (49). The stage of lactation can greatly influence the fatty acid composition of milk.

Mid lactation is defined as the period of production occurring between twelve and thirty weeks post-partum (79). During this time milk production continues to decline. The concentration of milk components is higher during mid lactation than during early lactation. The animals are in positive energy balance as feed intake has been elevated to accommodate the demands of milk production. When the animals are in positive energy balance, adipose tissue is not being mobilized to the same extent. However, during this time lipogenesis in adipose tissue is reduced in order to direct the dietary nutrients away from adipose tissue to the mammary gland. During this period and late lactation, C6 - C16 and C18 remain relatively stable (49).

Late lactation is defined as the period from approximately 30 weeks post partum to drying off which occurs at around 44 weeks (79). During this period, milk production continues to decline until the animal is dried off at around 305 days of lactation. At this time the milk is more concentrated so that the milk fat percentage is higher than during early or mid lactation. The animals are still in positive energy balance and are not experiencing lipolysis. As well, lipogenic activity in adipose tissue is higher than during mid lactation. As a result, lipid storage in adipose tissue is active as the animal regains its body condition.

1.3 - Milk Composition

Bovine milk is characterized by milk lipids that are primarily triglycerides (Table 1.1) (17). Of the fatty acids in milk, approximately 25 % are short and medium chain fatty acids which are synthesized in the mammary gland. The majority (64 %) of fatty acids in milk are saturated fatty acids while the remainder are primarily monounsaturated fatty ~~acids~~ (17). The action of ruminal biohydrogenation on dietary lipid results in a high degree of saturation of dietary fatty acids. This maintains unsaturated and polyunsaturated fatty acids at a low concentration in milk. Stearoyl-CoA desaturase is present in the mammary gland and its action results in the introduction of a cis Δ9 double bond in palmitic (C16:0) and stearic (C18:0) acids to yield palmitoleic (C16:1) and oleic (C18:1) acids.

Milk protein is largely made up of two major groups: casein and whey proteins. There are four kinds of casein proteins: α_1, α_2, β, and κ. The whey proteins are comprised of β-lactoglobulin, α-lactalbumin, lactoferrins and immunoglobulins. The other proteins that are represented in milk include a wide array of enzymes. Caseins are important for cheese production while the whey proteins are being examined for a variety of different uses including some bacteriocidal properties. In addition to protein, milk possesses nitrogen in the form of non-protein nitrogen (NPN). Amino acids are provided by diet and muscle turnover. Growth hormone stimulates muscle turnover and partitioning of dietary amino acids to the mammary gland to support the enhanced milk production.

Carbohydrates in milk are represented by lactose, monosaccharides, and oligosaccharides. Lactose is the most important of these components as it functions as the osmoregulator of milk. The production of lactose in the mammary gland directly determines the amount of milk that is being produced. In order to cause milk increases, lactose must first be increased. The ability of lactose to osmoregulate milk means that the synthesis of lactose and milk are closely associated. At the onset of lactation, progesterone declines which enables lactose to be synthesized. This action along with the release of prolactin, stimulates milk production. Following treatment with bGH, gluconeogenesis in the liver is stimulated (7) which increases the potential for lactose synthesis in the mammary gland. Glucose transporters are required for the uptake of glucose into the mammary gland as glucose can only be synthesized in the liver. Growth hormone stimulates milk production by increasing lactose synthesis. However, Zhao et al (80) demonstrated that bGH and bGRF treatment had no impact on glucose transporter GLUT 1 protein in the mammary gland. This suggests that the uptake of glucose into the mammary gland is not a rate limiting step in milk lactose synthesis. The remaining components of the solid fraction of milk are comprised of minerals, vitamins and salts.

1.4 - Milk Fat Synthesis

Bovine milk fat is derived from three sources: *de novo* synthesis in the mammary gland, dietary lipids and adipose tissue mobilization. Milk fat is estimated to be made up of 50 % fatty acids from de novo synthesis, 40 - 45 % from dietary sources and 10 % from adipose tissue mobilization (59). The animal's energy balance, stage of lactation and diet will greatly influence the extent to which any of these three sources are utilized for milk fat synthesis. Short and medium chain fatty acids are synthesized directly from acetate, β-hydroxybutyrate and lactate in the mammary gland through the action of acetyl-CoA carboxylase and fatty acid synthase. Other fatty acids are derived from dietary lipids and adipose tissue mobilization.

1.4.1 - De Novo Fatty Acid Synthesis in the Mammary Gland

The mammary gland is one of the two primary sites of lipogenesis in the ruminant animal. At parturition, the lipogenic genes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are induced in the mammary gland and the enzymes are produced in abundance (9, 60). The genes are induced to accommodate *de novo* synthesis that is required in the mammary gland for milk fat synthesis. Fatty acids of up to 16 or 18 carbons can then be synthesized. Long chain fatty acids cannot be synthesized in the mammary gland as there is no elongase activity in the mammary gland within the alveolar epithelial cells (17). Long chain fatty acids are provided by the diet and adipose tissue mobilization.

Fatty acids in the mammary gland are synthesized using acetate, β-hydroxybutyrate and lactate as the substrate (5, 17, 53). These are produced in the rumen as a result of microbial degradation of dietary carbohydrates. Acetate is the primary substrate and β-hydroxybutyrate is the second most common substrate for milk fat synthesis. β-hydroxybutyrate favours the synthesis of medium chain fatty acids (MCFA). The VFAs are converted to acetyl-CoA which can be used as the substrate for ACC to yield malonyl-CoA which is then used as the substrate for fatty acid synthesis. Unlike monogastrics, ruminants are not able to use the products of glycolysis, i.e. pyruvate, for milk fat synthesis. The pyruvate/malate cycle which is required for the utilization of pyruvate is not active in the ruminant mammary gland (64). Volatile fatty acids are the only substrate source utilized in *de novo* synthesis of fatty acids in the ruminant mammary gland.

Fatty acid synthesis occurs in the cytosol of mammary alveolar cells through the action of ACC and FAS. The majority of milk fats that are synthesized in the mammary gland are 4 - 16 carbons in length. Acetyl-CoA carboxylase is generally considered to be the rate limiting enzyme of fatty acid biosynthesis. The enzyme enables the conversion of acetyl-CoA to malonyl-CoA which is the substrate for fatty acid biosynthesis, and occurs via the addition of CO_2 from the donor HCO_3^-. The resultant malonyl-CoA is the substrate for FAS which can then synthesize fatty acids of up to 16 or sometimes 18 carbons in length.

In the first step of fatty acid biosynthesis, acetyl-CoA and malonyl-CoA are condensed through a series of reactions which are carried out by the multifunctional FAS enzyme. The result is an acyl chain of four carbons in length. The steps of fat synthesis following this initial step, involve the cyclic addition of two carbons from malonyl-CoA resulting in the acyl chain elongation up to a maximum of 16 carbons. Mammary gland FAS is unique in its ability to release SCFA and MCFA as activated CoA esters (17, 65, 66). This enables SCFA and MCFA to be released in a form which can then be incorporated into triglycerides and secreted in milk.

1.4.2 - Adipose Tissue Mobilization

In ruminants, unlike monogastrics, the liver is capable of virtually no lipogenic activity (19). This is primarily due to the fact that there is very little ACC activity in the liver (3). Also, the liver has only limited capacity for fatty acid secretion (19). For this reason, lipids derived from body stores are derived exclusively from adipose tissue.

Adipose tissue mobilization is mediated by two events: enhanced lipolysis and reduced lipogenesis. This process is stimulated in the early part of lactation when animals are in negative energy balance (5) which occurs when the energy requirements of lactation exceed those provided by the diet. The animals then mobilize their energy reserves in order to meet the energy demands of lactation.

De novo synthesis accounts for approximately 50 % of milk fatty acids which means that the remainder are provided by dietary lipids and adipose mobilization.

During lactation, insulin levels are reduced while GH levels are increased. Insulin normally stimulates lipogenesis in adipose tissue and inhibits lipolysis. Growth hormone acts upon adipose tissue via GH receptors and renders the tissue insensitive to insulin (45, 54, 73). In addition, epinephrine stimulated lipolysis is enhanced by GH (5). These combined events result in nutrients being directed away from adipose tissue to the mammary gland for milk fat synthesis. As well, the mobilization of lipid provides long chain fatty acids to the mammary gland for milk fat synthesis.

Epinephrine stimulates lipolysis by activating adenylate cyclase which results in a phosphorylation cascade that activates hormone sensitive lipase (HSL) (13). Hormone sensitive lipase then stimulates lipolysis resulting in the release of non-esterified fatty acids (NEFAs) and glycerol into the blood stream. The NEFAs can be transported to the mammary gland where they are taken up via a concentration gradient which is created as the fatty acids are incorporated into triglycerides in the tissue. The availability and uptake of NEFAs into the mammary gland is increased during early lactation (56) when the animals are in negative energy balance. This represents one of the two sources of long chain fatty acids in milk fat. The other source is dietary lipid.

1.4.3 - Utilization of Dietary Fat

Dietary lipids are the final source of milk fatty acids. Lipids are ingested as part of the forage and grain or in the form of lipid supplements such as tallow and oil seeds. Normally, lipid accounts for less than 5 % of the total diet (59). Higher levels of fat can have detrimental effects on the rumen environment and microbes, resulting in impaired fermentation. The majority of lipids are acted upon by rumen microbes and are biohydrogenated. The rumen bacteria first hydrolyse dietary triglycerides to yield fatty acids and glycerol. These fatty acids can then be acted upon by both bacterial and protozoan populations and are saturated. The extent of saturation depends upon the rumen conditions as this will determine what microbes are present in the rumen (59). The saturation of fatty acids in the rumen causes fatty acids reaching the small intestine, regardless of the degree of saturation of fatty acids in the diet, to be primarily saturated.

Recently, there has been a lot of work done on the feeding of protected lipids to the animal. The oil droplets can be protected in a variety of ways, e.g. formaldehyde or calcium ion protection, to yield a product which resists biohydrogenation in the rumen. These lipids are not acted upon by the ruminal microbial population which results in their being made available to the animal in their original form. The lipids are absorbed in the small intestine and can then go directly to the mammary gland. This enables the amount and type of unsaturated fatty acids present in milk to be altered.

Dietary fatty acids of C12 or less can be absorbed directly into the blood stream from the rumen. The remainder and all protected lipids are absorbed from the small intestine. Fatty acids are formed into micelles primarily in the jejunem of the small intestine. These are then taken up into the villous cells and are packaged as triglycerides into chylomicrons. The chylomicrons are released into the blood stream and can be transported to the mammary gland.

Dietary fatty acids are taken up into the mammary gland from chylomicrons following catalysis by lipoprotein lipase (LpL). Lipoprotein lipase catalyses the hydrolysis of triglycerides contained within the lipoprotein to release free fatty acids and monoglycerides. The fatty acids and monoglycerides are then taken up into the mammary gland through a concentration gradient which is created as the fatty acids are converted into triglycerides in the mammary gland. These triglycerides can then be released in the milk as a portion of milk fat.

Like the lipogenic genes, ACC and FAS, LpL is induced at the time of parturition in the mammary gland and demonstrates high activity during lactation (26, 35). Lipoprotein lipase activity increases during late gestation and remains high throughout lactation (42, 47). This enables dietary fatty acids which are packaged in chylomicrons, to be utilized by the mammary gland for milk fat synthesis.

1.4.4 - Synthesis of Unsaturated Milk Fatty Acids

The mammary gland possesses the ability to introduce a cis double bond at the C9 position of palmitic (C16:0) and stearic acid (C18:0). This enables the animal to maintain milk fluidity by ensuring a minimum percentage of unsaturated fatty acids. The ability of the animal to desaturate fatty acids in the mammary gland is an essential adaptation to rumen biohydrogenation.

Stearoyl-CoA desaturase (SCD) is induced at the onset of lactation and displays high activity. This enables palmitic acid which is generated from the three sources of milk fat, and stearic acid which is derived from adipose tissue mobilization and diet, to be desaturated at the Δ9 position. This maintains the percentage of unsaturated fatty acids at a concentration sufficient for maintaining milk fluidity. Normally, C16:1 and C18:1 account for approximately 30 % of milk fatty acids (17) with C18:1 being the predominant monounsaturated fatty acid. The majority of C16:1 and C18:1 are derived through the action of SCD in the mammary gland. This is an essential function in the ruminant animal for maintaining the fluidity of milk.

1.5 -Growth Hormone

Growth hormone is a naturally occurring hormone which belongs to the same family of hormones as prolactin and placental lactogen and is released from the anterior pituitary. The receptors for these hormones are also similar in structure and signal transduction (10). Growth hormone-releasing factor and somatostatin (SS) are produced in the hypothalamus and act directly on the anterior pituitary. Growth hormone is stored in secretory granules of the somatotroph cells of the anterior pituitary which are released via exocytosis (10). Growth hormone-releasing factor stimulates the release of GH from the pituitary while SS inhibits its release (7, 33, 48). As well, elevated serum GH levels feedback and inhibit the release of GRF and stimulate the release of SS which in turn inhibits the release of GH (10). Insulin-like growth factor I also acts in this way to prevent the secretion of GH when serum IGF-I levels are high (10). Growth hormone is involved in growth and in milk production and is elevated during lactation and in ruminants, is the major hormone involved in lactation (4, 74, 76). Prolactin stimulates the onset of lactation and GH is the primary hormone involved in the maintenance of lactation. The secondary role of prolactin was demonstrated by bromocriptine treatment which counteracts the action of prolactin. Bromocriptine treatment had no effect on milk production which indicated that in ruminants, prolactin is not the primary lactogenic hormone as it is in monogastrics (27).

Milk production is believed to be stimulated by GH indirectly rather than directly. Growth hormone stimulates the partitioning of nutrients from other tissues in the body, in particular adipose tissue, to the mammary gland (5, 29, 56).

In addition, IGF-I concentration is elevated by GH. Insulin-like growth factor I then acts directly on the mammary gland epithelial cells through IGF-I receptors and stimulates milk production (29, 61). Insulin-like growth factor I is also mitogenic and stimulates the division of the mammary epithelial cells which supports the increased milk production (29).

Nutrient partitioning from the adipose tissue is supported by a combination of processes. When animals are in positive energy balance it is generally accepted that nutrient partitioning away from adipose tissue to the mammary gland is supported by a reduction in adipose tissue lipogenesis. This prevents the precursors of lipid synthesis from being directed to adipose tissue for lipid storage and leaves them available for milk fat synthesis in the mammary gland. Growth hormone acts on adipose tissue directly by binding to GH receptors. This inhibits the action of insulin on adipose tissue which results in inhibition of lipogenesis (7, 51, 72). The end result is that the activity, mRNA and protein abundance of the lipogenic enzymes are significantly reduced by GH action (22, 45, 76).

In addition to inhibiting lipogenesis, epinephrine stimulated lipolysis is enhanced by GH action on adipose tissue (5, 22, 63, 72, 77, 78). Lipolysis occurs when animals are in negative energy balance (7, 45) and underlies adipose tissue mobilization. Growth hormone stimulates the action of HSL resulting in lipolysis of the adipose tissue depot. Lipolysis is also supported by the ability of GH to inhibit the anti-lipolytic action of adenosine and prostaglandin E_2 (72). The increased lipolysis releases NEFAs into the blood stream which are then transported to the mammary gland and are taken up for milk fat synthesis. Growth hormone also stimulates the uptake of NEFAs by the mammary gland (14, 55).

Growth hormone acts upon the liver to stimulate gluconeogenesis, glycogen mobilization and IGF-I release. This ensures that glucose is available to the mammary gland for lactose synthesis. During lactation, glucose is preferentially utilized by the mammary gland which enables the maintenance of milk production (5). As well, GH stimulates mobilization of protein from muscle to provide amino acids to the mammary gland as substrate for milk protein synthesis.

The natural galactopoietic action of GH has lead to its use as means of enhancing milk yield. Originally, an attempt was made using pituitary derived GH. However, the yield of GH that was obtained from pituitary glands did not make this a possibility for large scale use in milk production. The advent of recombinant DNA technology in the 1980's enabled the production of large amounts of relatively inexpensive recombinant bovine GH (bGH) (10). This has allowed wide scale investigation and the use of this product in commercial milk production.

The galactopoietic effect of bGH has been amply demonstrated (5, 8, 20, 21, 22, 27, 28, 44, 49, 68, 71, 76). Trials with bGH at different stages of lactation and at varying doses have demonstrated significant increases in milk yield. As a result of this, bGH is now being used in the United States in commercial production to stimulate milk yield. Recombinant bGH has been demonstrated to increase milk production mainly without altering milk composition (7, 20, 21, 37, 38, 49, 67) although there have been some reports of milk fat percentage increases (31, 45). In particular, milk fat percentage and composition are normally unaltered (2, 20, 37, 38, 49, 67). Therefore, although milk composition is generally unaltered the yield of milk components is enhanced as a result of elevated milk yield. This requires increased uptake and synthesis of fat, lactose and protein in the mammary gland. As milk yield is increased, the processes which are naturally induced or inhibited by GH during lactation are enhanced by exogenous bGH administration.

The action of GH and the ability of bGH to enhance milk production without dramatically altering milk composition makes GH an interesting hormone for examination. There is still much information to be gained about the exact nature of the action of GH on the mammary gland. While the direct evidence to date suggests that GH exerts its effects on the mammary gland indirectly, mRNA for GH receptors has been demonstrated (32) and IGF-I and IGF-II have been demonstrated to be ineffective in their ability to maintain milk production at the same level as GH. This is a complex issue which needs to be resolved through further investigation.

1.6 - *Acetyl-CoA Carboxylase*

Acetyl-CoA carboxylase is synthesized in lipogenic tissues such as adipose tissue, mammary gland and the liver of monogastrics. This enzyme carries out the first committed step to fatty acid synthesis which involves the carboxylation of acetyl-CoA to yield malonyl-CoA. This is the product of the following half reactions which comprise the rate limiting step of fatty acid synthesis.

(1) $\textbf{Enzyme-biotin} + \textbf{HCO}_3^- + \textbf{ATP} \longrightarrow \textbf{Enzyme-biotin-CO}_2 + \textbf{ADP} + \textbf{Pi}$

(2) $\textbf{Enzyme-biotin} + \textbf{acetyl-CoA} \longrightarrow \textbf{Enzyme-biotin} + \textbf{malonyl-CoA}$

There are two catalytic regions in ACC which catalyse these half reactions (34). The net result is that acetyl-CoA is carboxylated in an energy requiring reaction using bicarbonate as a donor to yield malonyl-CoA as a product.

Acetyl-CoA carboxylase is normally expressed in the adipose tissue of ruminant animals. Unlike monogastrics, ACC activity is extremely low or absent in the ruminant liver which has virtually no fatty acid biosynthetic or secretion capacity (3). At the onset of lactation, ACC is induced in the mammary gland in order to carry out *de novo* synthesis of fatty acids in the mammary gland for milk fat synthesis. This increase is associated with a rise in the total amount of ACC as well as the proportion which is in the active state (50). In addition to the stimulation of lipogenesis in the mammary gland at parturition, ACC activity, mRNA and protein abundance are reduced in adipose tissue (75). This ensures that substrate for milk fat synthesis will be preferentially directed to the mammary gland.

The regulation of ACC is complex. It is subject to hormonal and dietary regulation. Covalent modification and allosteric control allow the enzyme to be responsive to the physiological requirements of the animal. The enzyme is subject to both rapid and long term regulation. Rapid regulation is accomplished through covalent modification and allosteric feedback while long term regulation is exerted when gene transcription is altered.

The enzyme can be reversibly activated or inactivated through covalent modification. In this way, protein kinase A, cAMP and AMPK phosphorylate specific serine residues rendering the enzyme inactive (16, 57). Serine residues 77, 79 and 1200 can be reversibly phosphorylated. When the enzyme is phosphorylated it becomes inactive, but it can be re-activated by dephosphorylation. This process is determined by the physiological state of the animal and whether there is a requirement for fatty acid synthesis.

Allosteric feedback control is exerted by citrate and long chain fatty acyl-CoA (57). Citrate is a substrate which can be converted to acetyl-CoA through the removal of oxaloacetate. As a result, higher concentration of citrate stimulates acetyl-CoA carboxylase activity while low concentrations are inhibitory. In addition, the products of fatty acid biosynthesis, long chain fatty acyl-CoA inhibit ACC activity when the concentration is high. When concentration of the acyl-CoAs is low and there is sufficient substrate, fatty acid biosynthesis and ACC activity are stimulated.

Long term regulation is exerted at the level of transcription (40). Lactation is an excellent example of this process as the gene encoding ACC is induced at parturition. This gives rise to elevated mRNA concentration and activity in the mammary gland. Messenger RNA abundance, and therefore transcription, of ACC in adipose tissue is reduced. At the end of lactation, the opposite situation occurs when transcription of ACC being turned off in the mammary gland and upregulated in adipose tissue. This enables the animals to respond to the demand for milk fatty acids which is created by lactation.

During lactation, serum insulin concentration is reduced and ST concentration is increased. Insulin is a potent stimulator of adipose tissue ACC, but during lactation with its reduced concentration and the increased concentration of ST, the ability of insulin to stimulate ACC activity in adipose tissue is inhibited. This results in the activity of ACC being significantly reduced in adipose tissue during lactation. Recombinant bGH has been demonstrated to reduce ACC activity further in the adipose tissue of lactating cows and goats (22, 45, 77).

Acetyl-CoA carboxylase has 265 and 280 kDa protein isoforms which are tissue specific. The 280 kDa protein is found in heart and skeletal muscle and is insensitive to the nutritional status of the animal (34). The 265 kDa protein is found in adipose tissue, mammary gland, and the monogastric liver and is influenced by the nutritional status of the animal, and is therefore insulin responsive. In lipogenic tissues, fatty acid biosynthesis responds to the dietary availability of fatty acids and substrates of their synthesis.

The coding sequence of the gene for acetyl-CoA carboxylase has also been demonstrated to possess two promoters denoted PI and PII, in mice (18, 43, 60). It is likely that this is the case in ruminants also due to the high degree of conservation in the coding and amino acid sequence of this enzyme among species. The promoters give rise to multiple transcripts of varying sizes due to differential splicing of the five exons of ACC, and are active in a tissue specific manner also (18, 43, 60). Promoter I is active in adipose tissue, is insulin responsive, and is responsible for producing Class I transcripts which are found only in adipose tissue. During lactation, the activity of PI and the concentration of Class I transcripts are reduced (60). Promoter I is not active in the mammary gland. It is believed that PII is ubiquitous although it does not function to an appreciable degree in adipose tissue (43). It has been proposed that PII may function to provide the fatty acid requirements essential for cellular function (18). Promoter II is active in the mammary gland and can be induced so that the level of expression is enhanced during lactation (18, 60). This enables ACC protein to be synthesized in the mammary gland during lactation. Unlike PI, PII is not insulin responsive (43) which further supports the differential expression of ACC.

The existence of different promoters and isozymes enables ACC to respond to the demands of the specific tissues based upon the animal's physiological and nutritional status. This enables the animal to respond to its lipid requirements rapidly and over the long term. In this way, the animal has adapted to complex physiological processes in such a way that a gene may be turned off in adipose tissue and turned on in the mammary gland. This enables nutrients to be favourably directed to the mammary gland in order to support the increased demand for substrate required for lactation.

Acetyl-CoA carboxylase is the key enzyme involved in fatty acid synthesis. As the rate limiting enzyme, understanding the influence of GH on ACC action provides insight into its regulation in the bovine model during lactation. This is an essential element in the eventual goal of manipulating milk fat percentage to meet consumer demand.

*1.7 - **Fatty Acid Synthase***

Fatty acid synthase (FAS) is a multifunctional protein which carries out a series of reactions resulting in the formation of fatty acids of up to 16 or sometimes 18 carbons in length. The FAS enzyme exists as a native homodimer comprised of two 260 kDa polypeptide chains organized in a head to tail arrangement (34). The protein is arranged into a multifunctional globular arrangement with six catalytic activities and an acyl carrier protein (ACP) (34). Fatty acids are synthesized through cyclic elongation resulting in the sequential addition of two carbons to the acyl chain. In each cycle, all enzyme activities are required with the exception of the thioesterase. The cycles continue until the fatty acid has reached a maximum length of 16 carbons at which time the thioesterase catalyses its release from the enzyme.

The first step involves the condensation of acetyl-CoA and malonyl-CoA and yields a four carbon acyl chain. Both substrates are bound to the ACP and are brought into contact with the multifunctional enzyme so that the other catalytic functions can be carried out. Once the four carbon acyl chain has been generated, the cycle of reactions begins again with the malonyl moiety bound to the ACP acting as the two carbon donor. This continues until the thioesterase releases the final acyl-CoA from the enzyme (Figure 1.2).

The first reaction is catalysed by acyl transferase. In this step, acetyl-CoA and malonyl-CoA then malonyl-CoA alone, are bound to the enzyme at the 4' phosphopantetheine residue and CoA-SH is released. The second reaction involves β-ketoacyl synthase and results in the condensation of the ACP bound acetyl and malonyl groups and subsequently between the acyl chain and a malonyl group. β-ketoacyl reductase then acts on the condensed products to reduce them using NADPH + H^+ as a reducing agent to yield a reduced acyl chain and $NADP^+$. The fourth step involves the removal of a water molecule and is catalysed by β-ketoacyl dehydrase to yield a water molecule and an acyl chain possessing a double bond between C2 and C3. This is then reduced in the fifth step under catalysis by enoyl reductase using NADPH + H^+ as a reducing agent. The cycle is then repeated several times using malonyl-CoA as the two carbon donor until the acyl chain is released.

Normally, this process occurs until palmitoyl-CoA (C16:0) is released. In many species this can then be extended by elongase to produce long chain fatty acids. In the ruminant mammary gland, there is no elongase activity (17) which means that fatty acids which are greater than 16 carbons in length can only be derived from the diet or adipose tissue mobilization. As well, the bovine mammary gland FAS is unique in the ability of the thioesterase to release activated short and medium chain fatty acyl-CoA (C4 - C14) as well as palmitoyl-CoA (65, 66). This is especially important in the cow mammary gland as SCFA and MCFA comprise a significant portion of milk fat. Bovine mammary gland FAS also lacks substrate specificity which enables acetoacetyl-CoA and crotonyl-CoA to be utilized in addition to acetyl-CoA and malonyl-CoA as substrates for fatty acid synthesis (1). This enables fat synthesis to be carried out when acetyl-CoA is limiting.

Fatty acid synthase is induced in the mammary gland at parturition. It is also significantly reduced in adipose tissue at this time (74). In the same way as ACC, this enables the substrates for fat synthesis to be concentrated to the mammary gland in order to promote milk fat synthesis. During this state, lipogenesis in adipose tissue is suppressed so that lipid storage is not favoured. Fatty acid synthase is subject to hormonal and dietary regulation which can be exerted during transcription and translation (34, 55). Insulin normally stimulates FAS in adipose tissue, but the reduced insulin concentration and increased GH levels associated with lactation, result in the reduction of activity, protein and mRNA abundance in adipose tissue.

As in ACC, two promoters (PI and PII) have been identified in the FAS coding sequence (39). Promoter II is downstream from PI. This may also be true in the bovine due to the highly conserved nature of the FAS coding and amino acid sequence. It is possible that these function in a tissue specific manner similar to ACC which may in part determine the differential expression of FAS in mammary gland and adipose tissue during lactation.

Treatment with bGH has been demonstrated to further reduce FAS activity in adipose tissue (36, 45). The ability of GH to act directly on adipose tissue and not on mammary gland may also form the basis for the difference in action between these two tissues.

While *de novo* synthesis accounts for only a portion of milk fat synthesis, understanding the action of FAS is essential to the eventual manipulation of milk fat.

1.8 - Lipoprotein Lipase

Lipoprotein lipase (LpL) catalyses the hydrolysis of triglycerides contained within lipoproteins such as very low density lipoproteins (VLDL) and chylomicrons. In adipose tissue and muscle, LpL is synthesized in the parenchymal cells and is transported to the capillary endothelium where it is bound to the surface with heparin sulfate linkages (25). In mammary gland, LpL is synthesized in the epithelial cells of the mammary alveoli and is then transported to the capillary endothelium (11, 41).

At parturition, LpL is induced in the mammary gland and is reduced in adipose tissue (52). Exogenous bGH tends to enhance this process (46). However, bGH apparently has no effect on the activity of LpL in the mammary gland (46). During lactation, LpL activity in the mammary gland is high which increases the delivery of fatty acids from dietary and endogenous sources. In ruminant animals, the triglycerides being delivered to the mammary gland are mainly of dietary origin and are contained within chylomicrons as the ruminant liver has very little capacity for either fatty acid synthesis or VLDL release.

Dietary fatty acids are packaged into chylomicrons in the small intestine and are transported to the tissues of the body. During lactation, the concentration of chylomicrons which are directed to the mammary gland is enhanced and the triglyceride delivery to adipose tissue and muscle is reduced. This is reflected in the LpL activity in the peripheral tissues. This results in dietary triglycerides being preferentially directed to the mammary gland. Once the chylomicrons have reached the target tissue, LpL bound to the capillary endothelium is activated by apolipoprotein CII which is contained in the chylomicron (25). The triglycerides contained within the chylomicron are then hydrolysed to yield primarily free fatty acids and 3-monoglycerides (25). The free fatty acids and monoglycerides are then taken up into the target tissue via a concentration gradient which is created as the free fatty acids and monoglycerides are converted to triglycerides in the tissue (62). The triglycerides which are formed in the mammary gland are then utilized for milk fat production. This is one of the sources of long chain fatty acids and potentially unsaturated fatty acids in the milk fat of the ruminant animal.

Lipoprotein lipase is stimulated by insulin (23, 24). Nonesterified fatty acids are believed to inhibit LpL activity (25). The activity of LpL can be controlled at the level of transcription, translation, post translational modification, processing and secretion (23). This enables short and long term regulation of LpL. Although the activity of LpL in the lactating mammary gland of the mouse has been reported to be as much as 20 x times higher than in the non-lactating mammary gland, this change was not accompanied by equivalent increases in LpL mRNA abundance (42). This is a particularly interesting finding as it implies that mRNA abundance does not reflect the enzyme activity.

The serum insulin and GH concentration associated with lactation result in adipose tissue insulin insensitivity. For this reason, since LpL in adipose tissue is stimulated by insulin, LpL activity is reduced during lactation. Prolactin has been demonstrated to be the secondary regulator of LpL in rats. This could provide the basis of LpL stimulation in the mammary gland during lactation as prolactin acts directly upon the mammary gland. However, while prolactin is essential to lactation, in the ruminant animal the primary hormone involved in lactation is GH. As a result, it is likely that GH may have a role in the activation of LpL in the lactating mammary gland. However, Liesman et al (46) demonstrated that there was no effect on LpL activity in the mammary gland arising from bGH administration in spite of the fact that milk yield was increased without any changes in milk composition. This suggests that GH does not have a direct role in the activation of LpL, but rather exerts its effects through the suppression of its activity in adipose tissue. Insulin-like growth factor I may also play a role in the increase in activity of LpL in the mammary gland due to its structural similarity to insulin. The results of Liesman et al (46) would seem to contradict this statement though, since LpL activity was not increased even though IGF-I concentration was elevated following bGH treatment.

The potential for LpL to be inhibited by NEFAs may lead to a reduction in its activity during times when the animal is in negative energy balance. At this time, the animal experiences enhanced lipolysis as the energy intake of the animal has not yet caught up with the energy output in the milk. This forces the animal to mobilize its own body reserves resulting in higher serum concentration of NEFAs. This may lead to suppression of LpL activity in the mammary gland and adipose tissue when NEFA concentration is high. At this time, adipose tissue mobilization would then be the preferred source of long chain. However, when the animal is in positive energy balance and it is no longer necessary to mobilize the body reserves, the activity of LpL may be restored and the diet may then become the preferred source of long chain.

The activity of LpL in the lactating mammary gland and the suppression of action in adipose tissue, leads to delivery of fatty acids to the mammary gland. This ensures that dietary fatty acids are preferentially directed to the mammary gland in order to support milk fat synthesis. This represents one of the three routes of milk fat synthesis. This is also one of the ways in which long chain fatty acids can be delivered to the mammary gland.

1.9 - Stearoyl-CoA Desaturase

Stearoyl-CoA desaturase (SCD) is normally expressed in the small intestine, muscle and adipose tissue of the ruminant animal (12). There is no SCD activity in the liver of the ruminant animal although there is high activity in the monogastric model (69). At the onset of lactation, SCD is induced at high levels in the mammary gland (15).

Stearoyl-CoA desaturase exists in two forms, SCD1 and SCD2. Stearoyl-CoA desaturase I is the form found in the lactating mammary gland and adipose tissue. There are three major proteins which make up SCD - NADH-cytochrome b5 reductase, cytochrome b5 and the terminal desaturase. Through a transfer of electrons between these proteins, stearoyl-CoA (C18:0) and palmitoyl-CoA (C16:0) can be converted to oleoyl-CoA (C18:1) and palmitoleoyl-CoA (C16:1) respectively (15) (Figure 1.3). This transfer of electrons enables animals to introduce Δ9 double bonds into these fatty acids. Polyunsaturated fatty acids cannot be produced by animals and must be consumed in the diet. In the ruminant animal, these will only become available for milk production if they are protected from biohydrogenation in the rumen.

Stearoyl-CoA desaturase is subject to hormonal and dietary regulation. The terminal desaturase is the site of regulation and influences the total activity of the enzyme. During the dry period, insulin stimulates SCD activity. During lactation, as with the other enzymes examined, the depressed level of insulin and the GH induced insensitivity to insulin, result in the suppression of SCD activity, protein and mRNA abundance in adipose tissue. This is accompanied by an increase in activity, protein and mRNA abundance in the mammary gland. In addition, arachidonic acid and linolenic acid have been demonstrated to reduce SCD activity in lymphocytes (58, 62). Oleic and palmitoleic acids do not inhibit SCD activity.

Stearoyl-CoA desaturase is synthesized in the mammary gland alveolar epithelial cells. It is produced in the mammary gland in order to ensure a level of desaturation in milk fat. The animal has adapted this process in order to maintain the fluidity of the milk. Milk which is heavily concentrated with saturated fatty acids and has very few or no unsaturated fatty acids will be much more viscous and will require more energy for secretion. The action of SCD is particularly important in the ruminant animal as the fatty acids consumed in the diet are saturated by the rumen microbes. This reduces the supply of unsaturated fatty acids which are available for milk production.

Treatment with bGH has been demonstrated to have no effect on the amount of unsaturated fatty acids which are present in milk (2, 49). As a result, since the yield of milk is increased and the amount of unsaturated fatty acids present in milk is not altered by treatment, the yield of unsaturated fatty acids is increased. For this reason, it is likely that the activity of SCD in the mammary gland is increased to meet the demand for unsaturated fatty acids. In adipose tissue, however, bGH treatment is likely to further reduce SCD activity since it enhances the insensitivity of adipose tissue to insulin.

In order to eventually be able to manipulate milk fat composition or to select cows which have naturally higher levels of SCD activity, it is necessary to study this gene and the enzyme it encodes completely. There is a lot of potential for increasing the amount of unsaturated fatty acids which occur in milk fat naturally simply by selecting animals which produce higher levels of SCD. There is considerable consumer pressure to produce bovine milk products which have higher concentrations of the unsaturated fatty acids which may have health advantages over the saturated fatty acids. This would provide a means of enhancing the level of unsaturated fatty acids in milk.

Conclusion

Milk fat synthesis is a coordinated process requiring the balance of three sources of fatty acids. Understanding the regulation of these three pathways will enable us to ultimately change the composition and percentage of milk fat. Consumer trends indicate that people desire a dairy product which is lower in fat and contains more unsaturated fatty acids. Changes in the composition of milk can be achieved by altering feeding practices as well as through the selection of animals which are perceived to produce milk with desirable qualities. Understanding the way in which the genes and enzymes involved in the synthesis, uptake and desaturation of fatty acids are regulated, will enable us to recommend practices to help match composition to changing consumer demand. We are hopeful that the end result of this research will bring us closer to understanding the sources of regulation of milk fat synthesis regulation.

Table 1.1 - Lipid Composition of Bovine Milk (17)

Lipid Class	Percentage
Triglycerides	98 %
Diglycerides	0.36 %
Monoglycerides	0.027 %
Cholesterol Esters	trace
Cholesterol	0.31 %
Free Fatty Acids	0.27 %
Phospholipids	0.6 %

Figure 1.1 - Average Lactation Curve of the Bovine

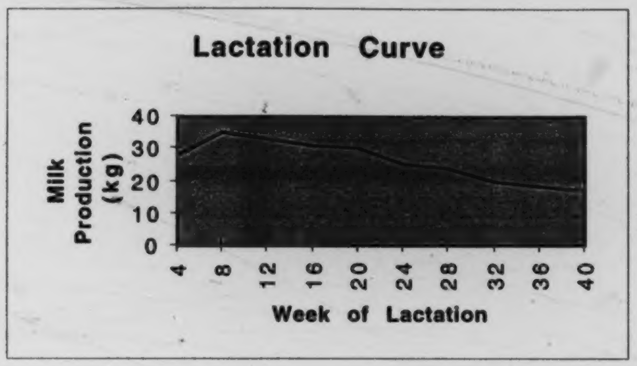
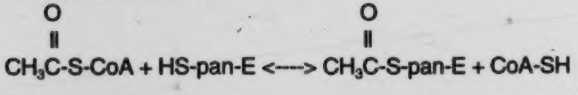


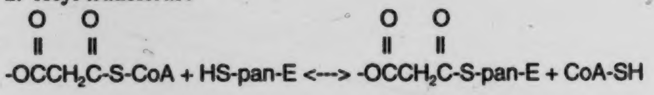
Figure 1.2 - Fatty Acid Synthesis (34)

1. Acyl transferase



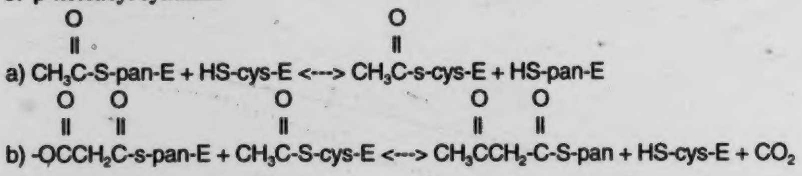
$$CH_3\overset{\overset{O}{\|}}{C}\text{-S-CoA} + \text{HS-pan-E} <\!\!-\!\!-\!\!\!> CH_3\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{CoA-SH}$$

2. Acyl transferase



$$\text{-O}\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}\text{-S-CoA} + \text{HS-pan-E} <\!\!-\!\!-\!\!\!> \text{-O}\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{CoA-SH}$$

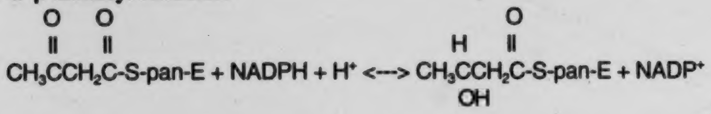
3. β-ketoacyl synthase



$$\text{a) } CH_3\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{HS-cys-E} <\!\!-\!\!-\!\!\!> CH_3\overset{\overset{O}{\|}}{C}\text{-s-cys-E} + \text{HS-pan-E}$$

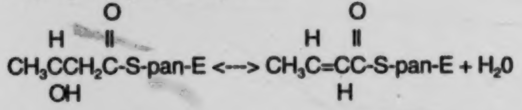
$$\text{b) -O}\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}\text{-s-pan-E} + CH_3\overset{\overset{O}{\|}}{C}\text{-S-cys-E} <\!\!-\!\!-\!\!\!> CH_3\overset{\overset{O}{\|}}{C}CH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-S-pan} + \text{HS-cys-E} + CO_2$$

4. β-ketoacyl reductase



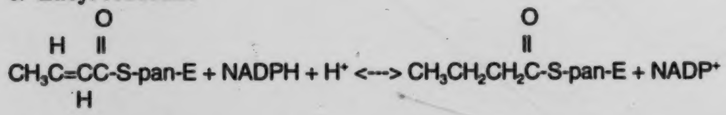
$$CH_3\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{NADPH} + H^+ <\!\!-\!\!-\!\!\!> CH_3\overset{H}{\underset{OH}{C}}CH_2\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{NADP}^+$$

5. β-hydroxyacyl dehydrase



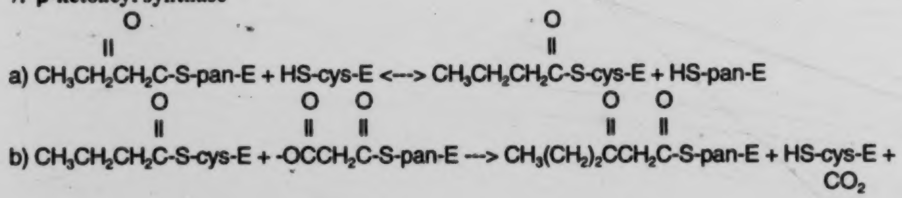
$$CH_3\overset{H}{\underset{OH}{C}}CH_2\overset{\overset{O}{\|}}{C}\text{-S-pan-E} <\!\!-\!\!-\!\!\!> CH_3\overset{H}{C}{=}\underset{H}{C}\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + H_20$$

6. Enoyl reductase



$$CH_3\overset{H}{C}{=}\underset{H}{C}\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{NADPH} + H^+ <\!\!-\!\!-\!\!\!> CH_3CH_2CH_2\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{NADP}^+$$

7. β-ketoacyl synthase



$$\text{a) } CH_3CH_2CH_2\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{HS-cys-E} <\!\!-\!\!-\!\!\!> CH_3CH_2CH_2\overset{\overset{O}{\|}}{C}\text{-S-cys-E} + \text{HS-pan-E}$$

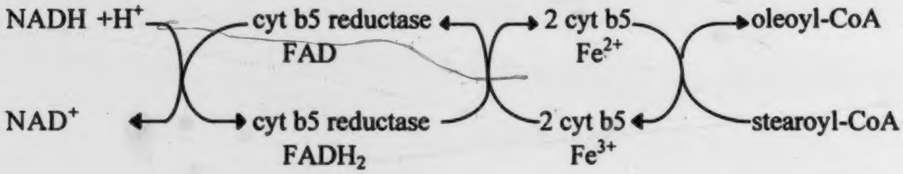
$$\text{b) } CH_3CH_2CH_2\overset{\overset{O}{\|}}{C}\text{-S-cys-E} + \text{-O}\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}\text{-S-pan-E} -\!\!-\!\!\!> CH_3(CH_2)_2\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}\text{-S-pan-E} + \text{HS-cys-E} + CO_2$$

8 - 10 Repeat reactions 4, 5 and 6.

11 - 30 Repeat reaction 3,4, 5 and 6 five times, with the molecule growing by two carbons each time (Fewer times if yielding shorter fatty acids than C16).

31. Thioesterase - to yield fatty acid.

Figure 1.3 - Fatty Acid Desaturation (58)


NADH +H$^+$
NAD$^+$
cyt b5 reductase FAD
cyt b5 reductase FADH$_2$
2 cyt b5 Fe^{2+}
2 cyt b5 Fe^{3+}
oleoyl-CoA
stearoyl-CoA

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Chapter 2

Influence of recombinant bovine growth hormone and growth hormone-releasing factor on acetyl-CoA carboxylase mRNA and protein abundance.

Introduction

Growth hormone (GH) is released from the anterior pituitary and is elevated during lactation (14). The release of GH from the anterior pituitary is tightly regulated by growth hormone releasing factor (GRF) which stimulates, and somatostatin (SS) which counteracts the release of GH (4, 17). In the bovine, GH is recognized as the key lactogenic hormone and is believed to act by elevating serum insulin-like growth factor I (IGF-I) (9, 14, 41, 46, 52) and favouring nutrient partitioning from other tissues to the mammary gland (2, 4, 13, 35, 41, 47, 49). Insulin-like growth factor I then acts directly upon mammary gland via IGF-I receptors. Although the message for GH receptors has been detected (16), GH has never been demonstrated to act directly upon the mammary gland.

The ability of administration of recombinantly derived bovine growth hormone (bGH) to enhance milk production has been amply demonstrated (5, 7, 11, 12, 18, 19, 22, 31, 42, 43, 49). Bovine GRF has been shown to increase the serum concentration of endogenous GH (19, 30), and to elicit increases in milk production equivalent to those achieved by bGH administration (4, 7).

The effects of bGH and bGRF on the composition of milk have also been well documented. Milk fat percentage and composition tend to be unaltered by either treatment (4, 7, 11, 18, 19, 31, 42, 43) although there are reports of milk fat percentage being increased by bGH treatment when cows are in early lactation (15, 26). The effects of exogenous and endogenous GH on lipogenesis in the mammary gland and adipose tissue have also been well examined in a number of species. Recombinant and endogenous GH inhibit lipogenesis (1, 25, 28, 46, 49) and enhance lipolysis in adipose tissue (2, 12, 26, 46, 48, 51). As well, the anti-lipolytic effect of molecules such as adenosine and prostaglandin E_2 is inhibited by GH (26). Finally, at the onset of lactation when endogenous GH levels are elevated, lipogenesis in the mammary gland is stimulated (1, 31, 33).

Acetyl-CoA carboxylase (ACC) catalyses the biotin dependent conversion of acetyl-CoA to malonyl-CoA. This is considered to be the rate limiting step of fatty acid synthesis. For this reason, its regulation may play a role in the action of bGH and bGRF on milk fat synthesis. Acetyl-CoA carboxylase is allosterically controlled and covalently modified and responds to both hormonal and metabolite stimuli (20, 36, 39). As well, transcription and translation of ACC are induced in the mammary gland at parturition while the message and protein concentration in adipose tissue are dramatically reduced (39). The existence of tissue specific promoters provides the basis for the differential expression during lactation. Growth hormone has been demonstrated to reduce the total activity of ACC in adipose tissue without changing the proportion of ACC in the active state (26, 48, 49). There are no reports in the literature on the influence of GH on mammary gland ACC in cattle.

The objective of this study was to examine the influence of continuous intravenous infusion of bGH and bGRF on the expression and synthesis of acetyl-CoA carboxylase (ACC) in the mammary gland and adipose tissue of primiparous Holstein cows. We hypothesized that the abundance of ACC message and protein would be increased in the mammary gland and decreased in adipose tissue as a result of bGH and bGRF treatment and that there would be no difference between the effects exerted by these treatments.

Materials and Methods

2.1 - Experimental Design

The animals used in this study were part of a larger study conducted at Michigan State University. Details of the study are provided in Binelli et al (4). In brief, 30 primiparous cows were assigned at random to one of ten blocks on the basis of parturition date. Within each block, one animal was treated with continuous intravenous infusion of 29 mg/day of recombinant bovine growth hormone (bGH) (Somavubove, The Upjohn Company, Kalamzoo, MI, USA). Another animal was treated with continuous intravenous infusion of 12 mg/day of recombinant bovine growth hormone releasing factor (1-45) homoserine lactone (bGRF) (The Upjohn Company). The last animal in each block received no treatment. The bGRF levels were designed to achieve similar circulating GH as that caused by bGH treatment and was based on the results of Dahl et al (9). Animals received treatment from 118 to 181 ± 1 d postpartum. On the final day of the treatment, animals were slaughtered, tissue was harvested, frozen on liquid nitrogen and stored at - 70 °C.

2.2 - Northern Blotting

2.2.1 - RNA Isolation

Total ribonucleic acid (RNA) was isolated from 200 mg of the mammary gland tissue of six animals from each of the control, bGH and bGRF treated groups using TRIzol (Gibco BRL / Life Technologies) and chloroform in a protocol modified from that described by Chomczynski (6) (Appendix 1). The resultant pellets were resuspended in 100 - 200 μl of sterile Milli-q water. Total RNA was isolated from four 200 mg samples of the adipose tissue of four animals from each treatment group. The pellets were resuspended in 25 - 50 μl of sterile Milli-q water then pooled. Total RNA was isolated from 200 mg of rat liver and bovine liver and was resuspended in 200 μl of sterile Milli-q water. The number of animals examined for mammary gland ACC differs from the number of animals examined for adipose tissue ACC as the adipose tissue sample of the remaining two animals was not available.

2.2.2 - RNA Quality and Quantity Determination

Ribonucleic acid was quantified by measuring its absorbance at 260 nm using a Gene Quant spectrophotometer (Pharmacia, Cambridge, England). Absorbance values of 0.1 - 0.8 were considered to be within the linear range of reading for this instrument.

The first indication of RNA quality was provided by measuring the absorbance of the RNA at 280 nm and taking the ratio of the absorbance at 260 nm: 280 nm. Values of 1.7 - 2.0 were deemed acceptable and were used for analysis. Ratios within this range indicate that the RNA has very little TRIzol contamination. The second determination of RNA quality was provided by running 5 μg of total RNA from each sample on a 1 % agarose - formaldehyde mini-gel for 1.5 h at 100 V in 1 x MOPS (Appendix 1). The gel was then photographed on a UV transilluminator with Polaroid 665 film. Samples which had a ratio of 2:1 for the ethidium bromide stained 18 and 28 s ribosomal RNA species were considered useful for further analysis.

2.2.3 - Detection Level Determination

In order to determine the RNA concentration required for ACC detection, a dosage gel of increasing amounts of RNA was analysed. Using a 1 % agarose - formaldehyde denaturing gel, RNA samples of 10 - 40 μg from fresh bovine mammary gland, bovine liver and rat liver were electrophoresed at 100 V for 5 h using 1 x MOPS as the buffer. Transcript size determination was achieved using

7.5 μg of a 0.24 - 9.49 kb molecular weight size marker (Gibco BRL / Life Technologies cat. # 15620 - 016).

The gel was photographed on a UV transillumator using Polaroid 665 film. The gel was placed on a capillary transfer apparatus and the RNA was transferred to the NitroPure nylon supported nitrocellulose (Micron Separations Inc.) for 18 - 20 h (Appendix 1). The membranes were baked at 80 °C for 2 h.

2.2.4 -Preparation of cDNA Probe

A 2.0 kb cDNA clone of a fragment of the coding sequence for ovine acetyl-CoA carboxylase (ACC) in pGEM7zf+, was obtained from Drs. Mike Barber and Maureen Travers at the Hannah Research Institute in Ayr, Scotland and was used to transform *Eschericia coli XLI blue.* Plasmid-insert DNA was then purified from the bacteria using a plasmid purification kit (Sigma Chemical Company, St. Louis, MO, USA). The cDNA insert was excised from the plasmid DNA using EcoRI. Insert cDNA was separated from plasmid DNA by running the excised DNA on a 1.2 % agarose gel for 2 h at 50 V in 1 x TAE. The insert cDNA was then purified from the agarose using a Gene Clean kit (Bio101, Vista, CA, USA) (Appendix 1). Five μl of the purified ACC cDNA was radiolabelled using a random primer DNA labelling kit (Gibco BRL / Life Technologies) and $\alpha[^{32}P]$dATP (Appendix 1).

2.2.5 - Hybridization, Washing and Autoradiography of Dosage Gel

The dosage membrane was prehybridized at 65 °C for 1 h in 25 ml of prewarmed prehybridization / hybridization solution (6 x SSPE, 0.5 % SDS, 5 x Denhardt's solution) (Appendix 1). The prehybridization solution was then replaced with hybridization solution containing the probe reaction and 100 μl of yeast tRNA. Hybridization was carried out at 65 °C for 16 - 18 h. The membrane was washed at room temperature in three 200 ml changes of low stringency wash (2 x SSPE, 0.1 % SDS) followed by a high stringency wash (0.1 x SSC, 0.1 % SDS) at 65 °C. The membrane was exposed to x-ray film (Kodak X-Omat AR) in a cassette with two intensifier screens for 1 d at -70 °C.

2.2.6 - Electrophoresis and Transfer of RNA of Treatment Samples

Thirty µg of the mammary gland total RNA from six animals of each treatment group were run on duplicate 1 % agarose - formaldehyde gels for 5 h at 100 V in 1 x MOPS. Twenty µg of total RNA isolated from the adipose tissue of four animals from each of the three treatment groups were run on duplicate 1 % agarose - formaldehyde gels at 100 V for 5 h in 1 x MOPS. Equivalent loading of RNA was corrected for by quantitating the ethidium bromide stained 18 s ribosomal RNA and dividing it from the volumes calculated by densitometry for the autoradiograph (6, 38, 43, 51, 52).

Following electrophoresis, the gels were photographed using Polaroid 665 film on a UV transilluminator. The gels were placed upon the capillary transfer apparatus and RNA was transferred to nylon supported nitrocellulose membrane (NitroPure, Micron Separations Inc.) for 18 - 20 h. RNA was then fixed to the membranes by baking at 80 °C for 2 h.

2.2.7 - Hybridization and Washing of the Sample Membranes

The cDNA probe was prepared as for the dosage gel using a random primer DNA labelling kit (Gibco BRL / Life Technologies) and α [^{32}P] dATP. Two membranes were prehybridized in 30 ml of pre-warmed hybridization/prehybridization solution (6 x SSPE, 0.5 % SDS, 5 x Denhardt's solution) at 65 °C for 1 h (Appendix 1). The solution was then replaced with 30 ml of fresh pre-warmed prehybridization/hybridization solution containing the radiolabelled probe reaction and 100 μl of yeast tRNA. Hybridization was carried out at 65 °C for 16 - 18 h. The membranes were washed in the same way as the dosage gel. The membranes were placed in an x-ray cassette with two intensifier screens and were exposed to x-ray film (Kodak X-Omat AR) for 1 - 3 d at - 70 °C. The films were then developed and the bands were quantitated using an imaging densitometer (BioRad Laboratories, Mississauga, ON).

2.3 - Western Blotting

2.3.1 - Protein Isolation

Approximately 1.0 g of mammary gland tissue from the same six animals, and from adipose tissue of four animals, of each treatment group, were homogenized in 4 °C homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 10 mM β-mercaptoethanol) (Appendix 1) containing 4 mg/ml leupeptin, antipain and pepstatin and 10 mM PMSF, with a 30 s burst using a Brinkmann Polytron power homogenizer. The homogenate was then ultracentrifuged in at 160 000 x g (48 000 rpm) for 1 h. The supernatant was stored at - 70 °C and the pellet was discarded. The supernatant derived from the adipose tissue protein isolation was centrifuged at 15 000 x g (10 000 rpm) for 20 minutes then was filtered through cheesecloth in order to remove the remaining lipid contamination.

2.3.2 - Protein Quantitation

Proteins were quantified using a bicinchoninic acid (BCA) Protein Assay Reagent kit (Pierce, Rockford, IL, USA). A standard curve was developed using bovine serum albumin (BSA). Concentration of protein was determined by measuring the absorbance at 540 nm and extrapolating from the standard curve.

2.3.3 - Gel Electrophoresis and Protein Transfer

Proteins were separated using SDS-PAGE with duplicate 4 % stacking - 5 % separating gels as described by Laemmli (24). Twenty-five μg of protein of the mammary gland samples were loaded along with a molecular weight marker (Rainbow 14.3 - 220 kDA molecular weight marker, Amersham International cat. # RPN 756) in 1 x denaturing sample buffer. The samples were separated on the gel 100 V for 2 h in 1 x electrode running buffer (5 mM Tris, 50 mM glycine, 0.02 % SDS). Using the same conditions, 60 μg of protein from the adipose tissue samples was separated.

Protein was transferred to nylon-supported nitrocellulose membrane (NitroPure, Micron Separations Inc.) using a current of 250 mA for 2 h in 4 °C Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol, 0.05 % SDS). The transfer was confirmed by staining the membrane for protein with Ponçeau S.

2.3.4 - ACC Protein Detection

Membranes were blocked for non-specific protein binding by soaking in 10 % Blotto/PBS/0.1 % Tween 20 on a shaker for 2 h. The mammary gland membrane was incubated with the streptavidin-horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at a concentration of 1:7500 for 1 h. Streptavidin recognizes the biotin molecule in ACC and can be used in place of a more specific antibody. The adipose tissue membrane was incubated with a concentration of 1:5000. The horseradish peroxidase was activated using ECL chemiluminescent reagent (Amersham International, Buckinghamshire, England) and the signal was detected with Hyperfilm ECL (Amersham International). The films were then developed and quantitated using an imaging densitometer (BioRad Laboratories, Mississauga, ON).

2.4 - Statistical Analysis

Results were analysed using SAS with the General Linear Models Procedure for least square means and the Student Neuman-Keuls test. Ydata, which was the set of raw data, was used as the dependent variable. Cow, gel and treatment variances were accounted for in the model. The error term was cow(treat). Results were expressed as least square means with standard error of difference (SED). Significance was set at $P < 0.10$.

Results

2.5 - Northern Hybridization Results

2.5.1 - Mammary Gland

Northern hybridization analysis of the 10.1 kb ACC message (Figure 2.1) revealed that there was no significant effect exerted by either bGH or bGRF treatment on the expression of ACC in mammary gland (Table 2.1). Least square means of arbitrary densitometric units (OD x mm^2) were 2.93, 3.63 and 4.16 for control, bGH and bGRF respectively and were not significantly different.

2.5.2 - Adipose Tissue

Northern hybridization analysis of the 10.1 kb ACC message (Figure 2.2) revealed that treatment with either bGH or bGRF resulted in suppression of the detectable message of ACC in adipose tissue (Table 2.2). The least squares mean abundance of the control values was 4.80 OD x mm^2.

2.6 - Western Blot Results

2.6.1 - Mammary Gland

Western blot analysis of the 265 and 280 kDa ACC protein isoforms (Figure 2.3) revealed that there was no effect of either bGH or bGRF on the synthesis of the ACC protein in the mammary gland (Table 2.3). Least square means of the control, bGH and bGRF were 13.98, 10.69 and 12.56 OD x mm^2 respectively and were not significantly different.

2.6.2 - Adipose Tissue

Western blot analysis of the adipose tissue samples revealed that the 265 kDa isoform (Figure 2.4) revealed that bGH ($p<0.004$) and bGRF ($p<0.004$) treatments significantly reduced its abundance when compared to control animals. Least squares means of the abundance of this protein were 6.61, 0.22, and 0.23 for control, bGH and bGRF treatment groups respectively (Table 2.4).

Discussion

Both bGH (3, 4, 7, 11, 12, 18, 23, 26, 27, 31, 43, 49) and bGRF (4, 10, 19) have been demonstrated to increase milk yield in dairy cows. Recombinant bGH is believed to stimulate milk yield by enhancing nutrient partitioning from other organs to the mammary gland (2, 3, 13, 14, 35, 49) and by elevating serum IGF-I (13, 40, 45, 51) which in turn acts directly and indirectly upon the mammary gland. The message for GH receptors has been detected in the mammary gland (16) which suggests that bGH may also act directly upon the mammary gland, but the latter has not been confirmed (17). Bovine GRF has been shown to increase serum GH levels by stimulating the release of endogenous GH from the anterior pituitary (4, 10, 19, 30). Recombinant bGH and bGRF have been demonstrated to have little or no effect on milk fat percentage or composition (3, 4, 7, 11, 18, 27, 31, 42) although there have been reports of increases in milk fat percentage when cows in early lactation are treated (15, 26).

Relative to controls, milk yield was elevated in cows treated with bGH or bGRF, but there was no treatment effect on milk fat percentage in the animals examined here (4). Milk yields were 29.1, 34.1, and 33.3 kg/d for the control, bGH and bGRF treated animals respectively (4). As milk fat percentage was unchanged and milk yield was increased by treatment, the yield of milk fat was enhanced, therefore, it was predicted that there would be an increase in the abundance of message and protein for lipogenic enzymes in the mammary gland. In particular, as ACC catalyses the rate limiting step of lipogenesis an increase in its synthesis and gene expression was expected. However, analysis of the 10.1 kb ACC transcript in the mammary gland of six animals from each group revealed that bGH and bGRF had no effect on the expression of the ACC gene in the mammary gland. This was supported by analysis of the 265 and 280 kDa isoforms of ACC protein in the mammary gland which were not altered by bGH or bGRF treatment. Liesman et al (28) reported a similar lack of effect of bGH or bGRF on lipogenesis with these animals as they failed to demonstrate increases in fat synthesis from acetate, fatty acid esterification or lipoprotein lipase activity in the mammary gland.

There are a number of possible explanations for the increased milk fat yield without an associated increase in ACC message or protein abundance. The activity of ACC can be controlled by covalent modification in which the serine 1200, 77 and 79 are phosphorylated rendering the enzyme inactive (20, 21, 36, 39). The proportion of ACC in the active to inactive state can be altered by hormonal treatment in both mammary gland and adipose tissue (26, 29, 49). It is possible that the proportion of ACC in the active state to that in the inactive state is increased by bGH and bGRF administration without altering the total amount of protein. Examination of this process as well as analysis of the enzyme activity is required, in order to more completely understand the status of ACC activity in the mammary gland. Also, protein turnover in mammary gland may be altered so that the enzyme half-life is increased in the mammary gland by the treatment rendering an increase in expression of the gene or protein to meet the needs of the increased milk fat yield unnecessary.

Binelli et al (4) also reported that there were elevated serum NEFA levels in the animals treated with bGH and bGRF and adipose tissue depots were significantly smaller than those of the control animals. Animals were calculated to be in positive energy balance although the treated animals were in a lower energy balance than the control animals. However, the elevated serum NEFA concentration of the treated animals when compared to the control animals, indicated that the animals were in negative energy balance (4). As well, Liesman et al (28) reported that hormone sensitive lipase (HSL) activity was elevated in the adipose tissue of animals from both treatment groups. Hormone sensitive lipase catalyses the lipolysis of triglycerides contained within adipose tissue. Additionally, adipose tissue depots were diminished by both treatments (4). This revealed that lipolysis was ongoing in the treated animals which is a characteristic of negative energy balance. Binelli et al (4) accounted for this by stating that the net energy of lactation (NE_L) of the diet was probably overestimated. This resulted in an inaccurate calculation of the animals' energy balance. The metabolic state of the adipose tissue revealed that the treated animals were actually in negative energy balance.

The elevated NEFA concentration reveals that a higher concentration is being delivered to the mammary gland as substrate for milk fat synthesis which then could be passively taken up from blood. We also found that lipogenesis in adipose tissue was profoundly reduced by both bGH and bGRF with no difference between the treatments. The abundance of the ACC message was suppressed below the level of detection in adipose tissue. In addition, analysis of the protein abundance in adipose tissue revealed that the 265 kDa ACC protein was significantly reduced by both bGH ($p<0.004$) and bGRF ($P<0.004$) treatment. Finally, Liesman et al (28) also reported a decrease in fat synthesis from acetate in adipose tissue. Acetyl-CoA carboxylase gene expression is under the control of two different promoters in rats (9, 22, 39) which may also form the foundation for differential expression during lactation in cows. Promoter I is active in adipose tissue and its activity is reduced at parturition when lipogenesis is suppressed (9, 39). Promoter II is ubiquitous (except for adipose tissue) and constitutive, but is induced at parturition in the mammary gland and is upregulated to accommodate the need for milk fat synthesis (22). Growth hormone may have a greater ability to act upon PI than on PII as it is believed that GH acts directly on adipose tissue to inhibit insulin stimulated lipogenesis (52). Growth hormone is not believed to act directly on the mammary gland which means that it may not act directly on ACC in the mammary gland, but rather to mediate its effects through IGF-I. This may account for the ability of GH to profoundly inhibit adipose tissue lipogenesis, but to not significantly stimulate lipogenesis in the mammary gland.

De novo synthesis of fatty acids in the mammary gland is reported to account for 50 % of all fatty acids in milk and is limited to short (SCFA) and medium chain fatty acids (MCFA) as the mammary gland lacks elongase, the enzyme required for extending fatty acids past palmitic acid (38). The remainder of SCFA and

MCFA, and all long chain fatty acids (LCFA), are provided by uptake of triglycerides and NEFAs from the blood. Ponce - Castaneda et al (39) determined that increased need for fatty acid synthesis during lactation in rats was met by increased *de novo* synthesis and uptake of fatty acids from blood. As ACC expression and synthesis were not affected by treatment, *de novo* synthesis does not appear to be enhanced by bGH and bGRF. The increased milk fat, therefore, may be accounted for by the increased uptake of fatty acids from the blood. Thus, the results provide support for the view that the additional milk fat production arising from bGH and bGRF treatment was due to increased incorporation of storage lipids in milk fat rather than elevated *de novo* synthesis of fat in the mammary gland.

The ability of bGH and bGRF to inhibit lipogenesis and to enhance lipolysis in adipose tissue ensures enhanced nutrient partitioning to the mammary gland. As lipogenesis is inhibited in adipose tissue, nutrients are directed preferentially to the mammary gland. Also, enhanced GH leads to the increased release of NEFAs as reported by Binelli et al (4). These factors combine to provide more substrate to the mammary gland.

Further research is required to determine the proportion of ACC protein in the active state to that in the inactive state. Also, ACC activity studies on fresh tissue would provide valuable information on the true level of de novo synthesis. Finally, an examination of the role of fatty acid synthase is necessary for an understanding of the impact of bGH and bGRF on lipogenesis in the mammary gland. These results are presented in Chapter 3.

Conclusion

Acetyl-CoA carboxylase message and protein abundance in the mammary gland are not affected by bGH and bGRF treatment. Recombinant bGH and bGRF cause a reduction in the abundance of ACC message in adipose tissue to below the level of detection. Our findings suggest that *de novo* synthesis may not be increased in the mammary gland. The increased substrate for milk fat synthesis may therefore be from the increased uptake of NEFAs.

Table 2.1 - Effect of bGH and bGRF on ACC mRNA Abundance in Mammary Gland (OD x mm^2)

Treatment	Least Squares Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	2.93	0.95	n/a	6
bGH	3.63	0.95	ns	6
bGRF	4.16	0.95	ns	6

* ns $P > 0.10$

Table 2.2 - Effect of bGH and bGRF on ACC mRNA Abundance in Adipose Tissue (OD x mm^2)

Treatment	Least Squares Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	4.8007^a	0.52	n/a	4
bGH	0^b	0.52	$p<0.0001$	4
bGRF	0^b	0.52	$p<0.0001$	4

* a, b superscript denotes significant difference

Table 2.3 - Effect of bGH and bGRF on ACC Protein Abundance in Mammary Gland (OD x mm^2)

Treatment	Least Squares Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	13.96	0.85	n/a	6
bGH	10.69	0.85	ns	6
bGRF	12.56	0.85	ns	6

* ns P > 0.10

Table 2.4 - Effect of bGH and bGRF on ACC Protein Abundance in Adipose Tissue (OD x mm^2)

Treatment	Least Squares Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	6.61^a	1.17	n/a	4
bGH	0.22^b	1.17	$p<0.004$	4
bGRF	0.23^b	1.17	$p<0.004$	4

* a, b superscript denotes significance

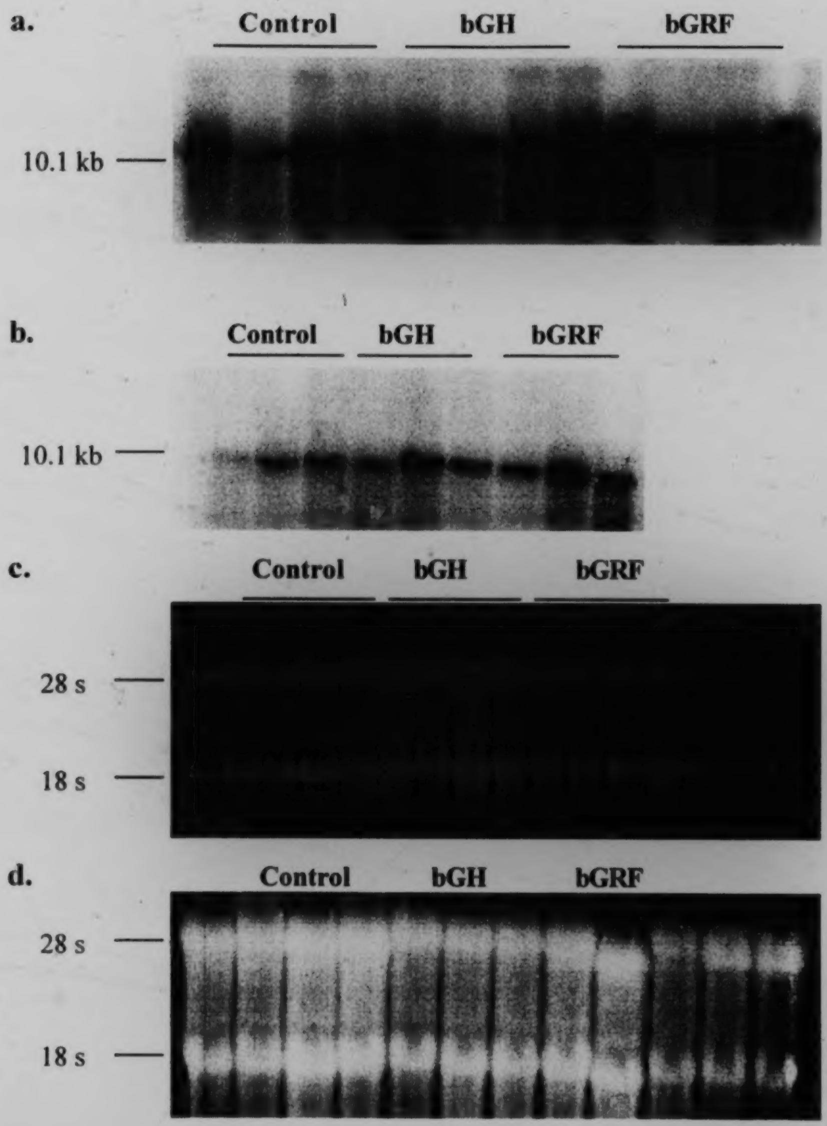
a.
Control
bGH
bGRF
10.1 kb
b.
Control
bGH
bGRF
10.1 kb
c.
Control
bGH
bGRF
28 s
18 s
d.
Control
bGH
bGRF
28 s
18 s

Figure 2.1 - Northern Blot of ACC Message in Mammary Gland
a) Northern blot of ACC mRNA in mammary gland of control, bGH and bGRF treated animals detected with 2.0 kb ovine cDNA probe, animals 1 - 4. b) Northern blot of ACC mRNA in mammary gland of control, bGH and bGRF treated animals detected with 2.0 kb ovine cDNA probe, animals 2, 5, 6. c) Ethidium bromide stained rRNA corresponding to a). d) Ethidium bromide stained rRNA corresponding to b).

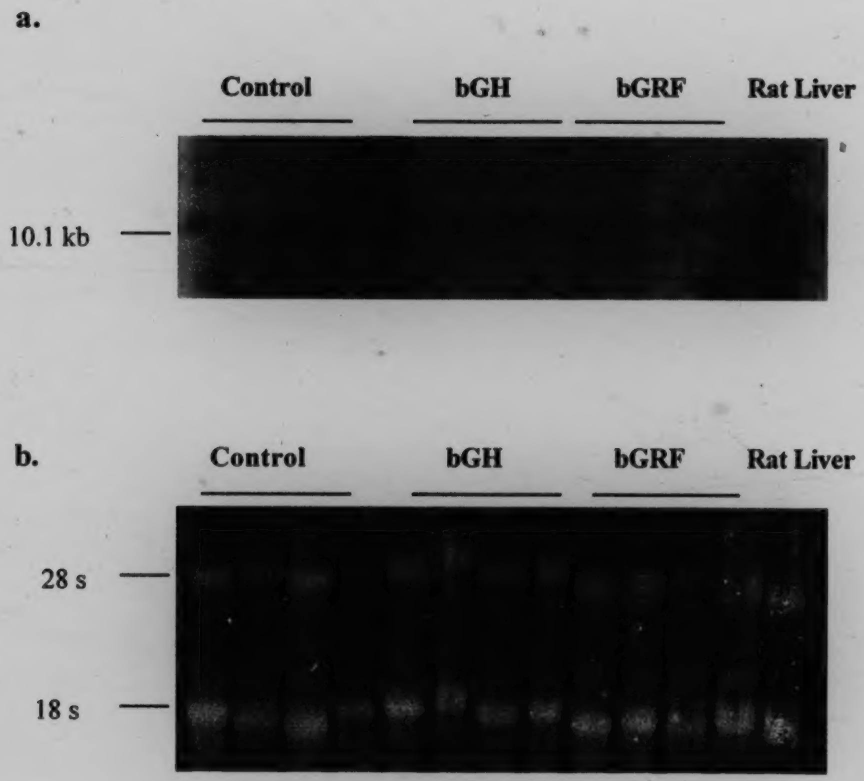
a.
Control
bGH
bGRF
Rat Liver
10.1 kb
b.
Control
bGH
bGRF
Rat Liver
28 s
18 s

Figure 2.2 - Northern Blot of ACC Message in Adipose Tissue
a) Northern blot of ACC mRNA in adipose tissue of control, bGH and bGRF treated animals detected with 2.0 kb ovine cDNA probe, animals 1 - 4. b) Ethidium bromide stained rRNA corresponding to a).

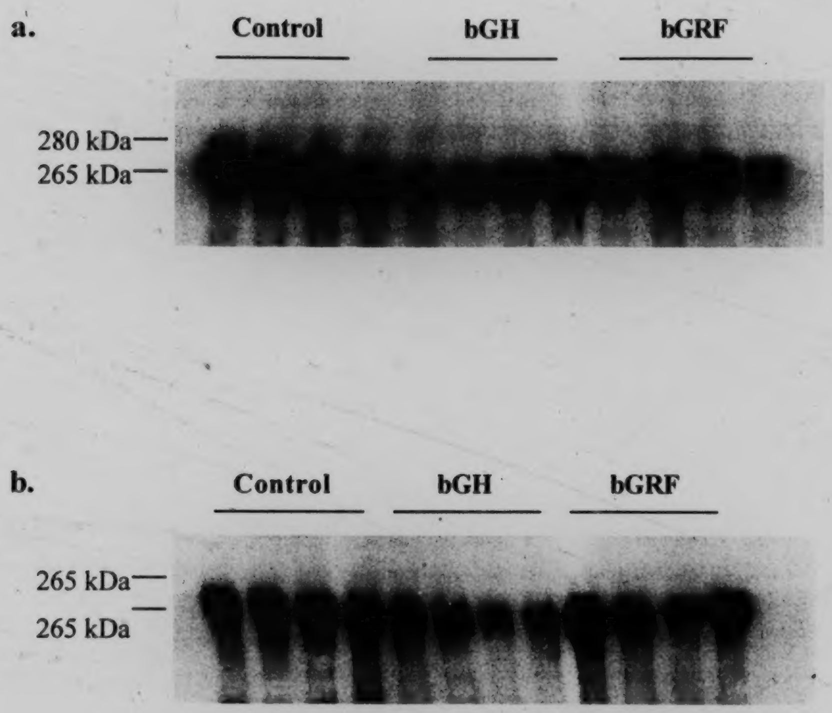
a.
Control
bGH
bGRF
280 kDa
265 kDa
b.
Control
bGH
bGRF
265 kDa
265 kDa

Figure 2.3 - Western Blot of ACC Protein in Mammary Gland
a) Western blot of ACC protein in mammary gland of control, bGH and bGRF treated animals detected with streptavidin-horseradish peroxidase, animals 1 - 4.
b) Western blot of ACC protein in mammary gland of control, bGH and bGRF treated animals detected with streptavidin-horseradish peroxidase, animals 3 - 6.

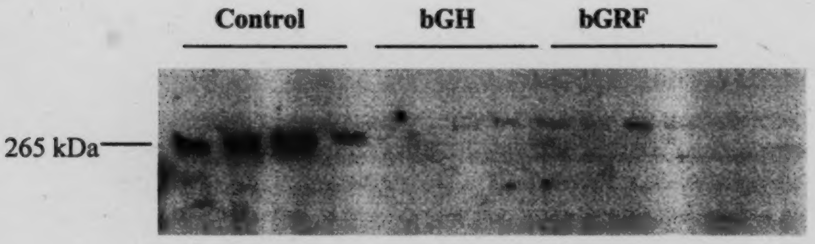
Control
bGH
bGRF
265 kDa

Figure 2.4 - Western Blot of ACC Protein in Adipose Tissue
Western blot of ACC protein in adipose tissue of control, bGH and bGRF treated animals detected with streptavidin-horseradish peroxidase, animals 1 - 4

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Chapter 3

Influence of recombinant bovine growth hormone and growth hormone-releasing factor on the mRNA and protein abundance of fatty acid synthase.

Introduction

Lactation is a complex process mediated by hormonal changes. Growth hormone (GH) is known to be the major lactogenic hormone in ruminants (4). Endogenous GH is released from the anterior pituitary under the control of growth hormone releasing factor (GRF) which positively affects its release and somatostatin (SS) which inhibits its release (4). Since the advent of recombinant DNA technology it is now possible to generate large quantities of recombinant bovine growth hormone (bGH) and growth hormone releasing factor (bGRF) thus facilitating the use of these hormones in commercial milk production. The ability of both bGH (3, 4, 5, 9, 10, 14, 15, 22, 23, 24, 26, 27, 29, 49) and bGRF (3, 9, 10, 14, 23, 27) to enhance milk production has been demonstrated. Recombinant bGH is believed to enhance milk production through increased nutrient partitioning (1, 2, 21, 37) and elevated serum IGF-I levels (45). Bovine GRF acts by increasing the secretion of endogenous GH (5, 9, 23).

Milk fat composition and percentage tend to be unaltered by bGH and bGRF administration (4, 5, 9, 15, 22, 23, 29, 42, 43), but there have been some reports of increases in milk fat percentage in early lactation cows (17, 26). The enhanced fatty acid supply necessary to accommodate the increased milk fat yield, may come from the diet, adipose tissue mobilization or *de novo* fatty acid synthesis in the mammary gland. Growth hormone alters adipose tissue metabolism by inhibiting insulin stimulated lipogenesis (4, 16, 26, 30, 39, 46, 47, 50) and enhancing lipolysis (1, 4, 13, 26, 31, 51). This ensures that dietary nutrients are not stored in adipose tissue, but are preferentially directed to the mammary gland.

While this is a normal process in lactation, bGH and bGRF increase the extent of nutrient partitioning to support enhanced milk production. Growth hormone is believed to act upon adipose tissue directly through GH receptors, but while mRNA for GH receptors has been demonstrated in mammary gland epithelial cells (19) the ability of GH to act directly upon mammary gland has not been demonstrated. Insulin-like growth factor I (IGF-I) is believed to mediate the effects of GH on the mammary gland via IGF-I receptors.

Fatty acid synthase is one of the key enzymes involved in the synthesis of fatty acids in lipogenic tissues. The native form of this protein is a dimer of 260 kDA polypeptide chains arranged in a head to tail fashion which are organized into globular multifunctional domains (20, 40, 52). The protein has six catalytic activities and an acyl carrier protein which together catalyse the synthesis of palmitic acid through the sequential addition of two carbons. The mammary gland form of FAS is unique in its ability to also produce short (SCFA) and medium chain fatty acids (MCFA) (40, 41). This is made possible through the use of acyl transferase which has chain specificity for shorter acyl chains as well (40). This allows SCFA and MCFA to equilibrate between the enzyme and CoA bound forms of the fatty acids which enables their incorporation into triglycerides. Mammary gland FAS is induced at parturition and is subject to hormonal and nutritional regulation (7).

Growth hormone has been demonstrated to reduce the abundance of FAS mRNA and protein in the adipose tissue of pigs (21, 43), sheep (48). In cattle GH has been shown to reduce FAS activity (26). An insulin responsive element in the sequence of FAS allows insulin to stimulate FAS synthesis (12, 18, 34), but when animals are treated with bGH or bGRF the ability of insulin to stimulate FAS synthesis in adipose tissue is inhibited (11). In the mammary gland of rats, GH has been demonstrated to increase FAS mRNA abundance (1).

In order to provide a more clear understanding of the effect of bGH and bGRF on milk fat synthesis, it is necessary to elucidate the direct effect of bGH and bGRF on adipose tissue and mammary gland fatty acid metabolism. Therefore, the objective of this experiment was to determine the effect of bGH and bGRF on the abundance of FAS mRNA and protein in the mammary gland and adipose tissue. We hypothesized that both bGH and bGRF treatment would result in an elevation of FAS mRNA and protein abundance in mammary gland and a decrease in mRNA abundance in adipose tissue.

Materials and Methods

3.1 -. Experimental Design

As described in Chapter 2, 30 primiparous Holstein cows were assigned to one of ten blocks on the basis of parturition date. Within each block, one animal was treated with continuous intravenous infusion of 29 mg/day of recombinant bovine growth hormone (Somavubove, The Upjohn Company, Kalamzoo, MI, USA). Another animal was treated with continuous intravenous infusion of 12 mg/day of recombinant bovine growth-hormone releasing factor (1-45) homoserine lactone (The Upjohn Company). The last animal in each block received no treatment and served as the control. The bGH and bGRF levels were calculated to produce similar circulating bGH levels based upon a previous experiment by Dahl et al, 1993 (10). Animals received treatment from 118 to 181 ± 1 d postpartum. On the final day of the treatment animals were slaughtered. The tissue was harvested, snap frozen in liquid nitrogen and stored at - 70 °C.

3.2 - Northern Blotting

3.2.1 - RNA Isolation

Total ribonucleic acid (RNA) was isolated from 200 mg of the mammary gland tissue of six animals from each of the control, bGH and bGRF treatment groups using TRIzol (Gibco/BRL) and chloroform extraction with isopropanol precipitation in a protocol modified from that of Chomczynski (8) (Appendix 1). The RNA was then resuspended in 100 µl of sterile Milli-q water. Total RNA was also isolated from four 200 mg samples of adipose tissue from four animals of each of the control, bGH and bGRF treatment groups. The pellets were resuspended in 25 µl of sterile Milli-q water, then pooled. Total RNA was also isolated from 200 mg of bovine liver from untreated animals and rat liver and was resuspended in 200 µl of sterile Milli-q water. The numbers of animals differ between tissues as the adipose tissue samples were not available for all six animals.

3.2.2 - RNA Quantitation and Quality Determination

Ribonculeic acid was quantified using a Gene Quant spectrophotometer (Pharmacia, Cambridge, UK). The absorbance of the sample at 260 nm was used to determine the concentration of the sample (Appendix 1). Samples which had absorbances of 0.1 - 0.8 were determined to be within the linear range for accuracy of this instrument.

The first indication of RNA quality is provided by determining the ratio of the absorbance of the sample at 260 nm:280 nm. Values between 1.7 - 2.0 were considered to be of sufficient purity for further analysis. The second determination of quality was provided by running 5 µg of each sample on a 1 % agarose-formaldehyde. Samples that did not demonstrate degradation and had a ratio of 2:1 for the ethidium bromide stained 28 s and 18 s ribosomal RNA species, were used for further analysis.

3.2.3 - Detection Level Determination

In order to determine the amount of total RNA that was required for the detection of the FAS message with rat FAS cDNA, a dosage gel of increasing quantities of RNA was run. Ten - 40 µg of total RNA from bovine mammary gland, bovine liver and rat liver were run on a 1 % agarose-formaldehyde gel along with an RNA molecular weight marker (0.24 - 9.49 kb, GibcoBRL / Life Technologies cat # 15620-016) for transcript size determination. The samples were electrophoresed for 5 h at 100 V with 1 x MOPS as the buffer.

Following electrophoresis, the gel was checked for RNA integrity by visualizing ethidium bromide stain with a UV transilluminator, and were photographed with Polaroid 665 film. The RNA was transferred to nylon supported nitrocellulose membrane (NitroPure, Micron Separations Inc.) by capillary transfer for 18 - 20 h, then was fixed to the membrane by baking at 80 °C for 2 h.

3.2.4 - cDNA Probe Preparation

A 2.5 kb cDNA clone of a fragment of the rat fatty acid synthase coding sequence was obtained from Dr. Stuart Smith of the Children's Hospital Oakland Research Institute, Oakland, CA., USA, and was used to transform *Eschericia coli XLI blue*. Amplified plasmid DNA was isolated using a DNA purification kit (PlasmidPURE DNA Miniprep kit, Sigma Chemical Company, St. Louis, MO, USA).

Fatty acid synthase insert cDNA was excised from plasmid DNA using EcoRI and XbaI. The insert cDNA was then separated from the plasmid DNA by running it on a 1.2 % agarose gel for 2 h at 50 V in 1 x TAE. The cDNA was then eluted from the gel using a Gene Clean kit (Bio101, Vista, CA, USA). The FAS cDNA insert was labelled using α [^{32}P]dATP and a random primer DNA labelling kit (GibcoBRL / Life Technologies).

3.2.5 - Hybridization, Washing and Autoradiography of the Dosage Gel

The dosage membrane was prehybridized in 25 ml of pre-warmed prehybridization/hybridization solution (6 x SSPE, 0.5 % SDS, 5 x Denhardt's solution) at 65 °C for 1 h. The prehybridization solution was then replaced with the same volume of pre-warmed prehybridization/hybridization solution containing the probe reaction and 100 µl of yeast tRNA (GibcoBRL / Life Technologies). Hybridization was carried out for 16 - 18 h at 65 °C. The membrane was washed in three 200 ml changes of low stringency wash (2 x SSPE, 0.1 % SDS) at room temperature. This was followed by one 200 ml change of the high stringency wash (0.1 x SSC, 0.1 % SDS) at 65 °C for 5 min. Following washing, the membrane was placed in an x-ray cassette with two intensifier screens and a Kodak X-Omat AR film. The film was exposed for 1 d at - 70 °C then developed.

3.2.6 - Electrophoresis and Transfer of Treated Samples

Twenty µg of total RNA from the mammary gland of each of the six animals from the three treatment groups, were run on duplicate 1 % agarose - formaldehyde gels. Samples were run for 5 h at 100 V in 1 x MOPS. Twenty µg of total RNA isolated from the adipose tissue of four animals from each treatment group were run on duplicate 1 % agarose - formaldehyde gels. Samples were electrophoresed at 100 V for 5 h in 1 x MOPS.

Following electrophoresis, the integrity of the RNA was examined on the UV transilluminator and the gel was photographed using Polaroid 665 film. The RNA was then transferred by capillary transfer to a nylon-supported nitrocellulose membrane (NitroPure, Micron Separations Inc.) for 18 - 20 h. The membrane was photographed once more and was baked at 80 °C for 2 h.

3.2.7 - Hybridization of Treated Samples

Insert cDNA isolation and probe preparation were performed as for the dosage gel. Two membranes were incubated together at 65 °C for 1 h in 30 ml of prewarmed prehybridization/hybridization solution. The prehybridization solution was then replaced with 30 ml of fresh prewarmed solution containing the probe reaction and 100 µl of yeast tRNA (Gibco BRL / Life Technologies). The membranes were then hybridized at 65 °C for 16 - 18 h. The washing steps were carried out as described for the dosage gel with the same volumes of solution.

The FAS message was detected in the same way as for the dosage gel. The signal was then quantified using an imaging densitometer (BioRad Laboratories, Mississauga, ON). Equal loading of RNA was corrected by quantifying the ethidium bromide stained 18 s ribosomal RNA and dividing it from the quantified autoradiography (7, 35, 37, 43, 52, 53). Values of densitometric analysis were expressed in arbitrary units (OD x mm^2).

3.3 - Western Blotting

3.3.1 - Protein Isolation

Protein was isolated from 1.0 g of mammary gland tissue of the six animals and from the adipose tissue of four animals from each of the three treatment groups. Tissue was homogenized on ice in 4 ° C homogenization buffer (50 mM Tris-HCl pH 7.5, 0.25 M sucrose, 1 mM EDTA, 10 mM β-mercaptoethanol) containing 4 mg/ml aprotinin, antipain and leupeptin as well as 0.5 mM PMSF, using a Brinkmann Polytron power homogenizer for a 30 s burst. The homogenate was then centrifuged at 160 000 x g (48 000 rpm) for 1 h. The supernatant was then stored at - 70 °C and the pellet was discarded. The supernatant from the adipose tissue samples was subjected to an additional centrifugation step at 15 000 x g (10 000 rpm) for 20 minutes then was filtered through cheesecloth in order to remove the residual lipid contamination. The supernatant was then also stored at -70 °C.

3.3.2 - Protein Quantitation

Protein was quantified using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA, cat # 23225). A standard curve of concentration was developed using bovine serum albumin (BSA). The absorbance of the protein was measured at 540 nm and was used to determine the concentration of the protein by extrapolating from the standard curve.

3.3.3 - Gel Electrophoresis and Antibody Detection

Twenty five µg each mammary gland sample and 60 µg of each adipose tissue sample were run on duplicate 4 % stacking - 5 % separating gels as described by Laemmli (25) along with a molecular weight marker (Rainbow 14.3 - 220 kDa molecular weight marker, Amersham International cat.# RPN 756). Electrophoresis was carried out at 95 V for 2 h in 1 x electrode running buffer (5 mM Tris, 50 mM glycine, 0.02 % SDS). Protein was then transferred to a nylon supported nitrocellulose membrane (NitroPure, Micron Separations Inc.) at 250 mA for 2 h with 4 °C Towbin's buffer (25 mM Tris, 192 mM glycine, 20 %

methanol, 0.005 % SDS). Transfer of protein was then confirmed using Ponçeau S.

Mammary gland membranes were incubated with sheep-anti-rat FAS/ACL (generously provided by Dr. Lee Witters at Dartmouth Medical College, Hanover, NH, USA) for 1 h at a dilution of 1:4000 in TBST. Adipose tissue membranes were incubated with the antibody at a concentration of 1:2500. The membranes were then incubated with secondary antibody anti-sheep IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, cat # 313-035-003) at a dilution of 1:7500 for mammary gland and adipose tissue in TBST for 1 hour. The horseradish peroxidase was activated using ECL chemiluminescent detection agent (Amersham International cat # RAN 2106). The membranes were exposed to Hyperfilm ECL film (Amersham International) for 15 - 30 s, then developed. The signals were quantified using an imaging densitometer (BioRad Laboratories, Mississauga, ON) and were expressed in arbitrary units (OD x mm^2).

3.4 - Statistical Analysis

Statistical analysis was performed using the General Linear Model of least square means and the Student Neuman-Keuls test. The model included treatment, cow and gel variances. Ydata was the term used to describe the raw data set and was set as the dependent variable. The error term was cow(treat). Results were expressed as least square means with SED. Significance was set at $P < 0.10$.

Results

3.5 - Northern Blot Results

3.5.1 - Mammary Gland

Quantitation of the 9.3 kb FAS message in mammary gland (Figure 3.1) revealed that both bGH ($p<0.01$) and bGRF ($p<0.03$) significantly increased FAS mRNA abundance (Table 3.1). Least square means expressed in arbitrary densitometric units of measure (OD x mm^2) were 9.42, 16.55 and 15.56 for control, bGH and bGRF respectively.

3.5.2 - Adipose Tissue

Treating animals with bGH and bGRF was found to reduce the 9.3 kb FAS message (Figure 3.2) in adipose tissue to below the level of detection (Table 3.2). The least squares mean of the control animals was 4.22 OD x mm^2 and differed significantly from both bGH ($p<0.002$) and bGRF ($p<0.002$) treated animals.

3.6 - Western Blot Analysis

3.6.1 - Mammary Gland

Western blot analysis of the 260 kDa denatured FAS protein (Figure 3.3), revealed that there was no significant effect of either bGH or bGRF on FAS protein abundance in the mammary gland (Table 3.3). The least squares means were 8.81, 9.29, and 9.48 OD x mm^2 for the control, bGH and bGRF groups respectively.

3.6.2 - Adipose Tissue

Analysis of the FAS protein abundance in adipose tissue revealed that both bGH ($p<0.0062$) and bGRF ($p<0.0054$) caused a significant reduction. The least squares means were 6.92, 0.71 and 0.55 OD x mm^2 for the control, bGH and bGRF treated animals respectively (Table 3.4).

Discussion

The galactopoietic effects of bGH and bGRF have been demonstrated previously. Similar results were reported by Binelli et al (5) for the animals used in this study. Milk production was significantly higher and milk composition was unaltered as a result of bGH and bGRF treatment. Yields of milk constituents, in particular milk fat, were therefore also significantly increased.

Increased milk fat yield would suggest that there is increased uptake of nutrients and *de novo* synthesis of fatty acids in the mammary gland. To elucidate this process, we examined the impact of bGH and bGRF on the expression and synthesis of FAS in the mammary gland. Analysis of gene expression revealed that both bGH and bGRF significantly increased the mRNA abundance of FAS in the mammary gland to 16.55 ($p<0.01$) and 15.56 ($p<0.03$) OD x mm^2 respectively as compared to 9.42 for controls. However, protein abundance was not altered by either treatment. This observation suggests that the activity of FAS in the mammary gland was unaffected by treatment since the amount of protein translated was similar across treatments. The level of protein synthesis in the mammary gland implies that there is no increase in the level of *de novo* fatty acid synthesis in the mammary gland in spite of the increased milk fat yield. The disparity between the increase in mRNA abundance and the lack of change in protein abundance, suggests that the relationship between mRNA and protein abundance in the mammary gland may not be directly correlated. Fatty acid synthase is known to be controlled at both the level of transcription and of translation (20) and is influenced by both hormonal and nutritional stimuli (7). It is possible that transcription and translation are differentially influenced by the indirect effects of bGH and bGRF. However, it is also possible that protein turnover is altered by treatment so that synthesized FAS enzyme has a longer half life. The results of Liesman et al (28) indicate the fat synthesis from acetate was not altered by either treatment. This suggests that the activity of the enzymes involved in milk fatty acid synthesis is not increased by either treatment.

The absence of treatment effects on de novo fatty acid synthesis in the mammary gland is also supported by our previous findings (Chapter 2) in which we demonstrated that there was no effect of bGH or bGRF treatment on abundance of ACC mRNA or protein in mammary gland. As ACC is the rate limiting enzyme of fatty acid synthesis and there is no effect of treatment on this enzyme, this provides strong supporting evidence of a lack of treatment effect on *de novo* synthesis of fatty acids in the mammary gland. The data for FAS reported here supports this finding and suggests that de novo synthesis in treated animals was similar to that of the control animals. In addition, ACC provides the malonyl-CoA substrate to FAS for fatty acid synthesis. As the amount of ACC protein was not increased, the assumption can be made that substrate supply was not increased by treatment.

Using animals from this experiment, Liesman et al (28) reported similar findings for this group of animals. They examined the synthesis of fatty acids from acetate in the mammary gland and adipose tissue and determined that there was no effect of either treatment on fat synthesis in the mammary gland. However, fat synthesis from acetate was dramatically reduced in adipose tissue. These results indicate that while lipogenesis in the mammary gland is not increased, nutrients are being directed away from adipose tissue to the mammary gland in order to support milk fat synthesis.

Our results demonstrated that mRNA abundance of FAS in adipose tissue was reduced below the level of detection by both treatments. As well, FAS protein abundance was significantly reduced by bGH ($p<0.0062$) and bGRF ($p<0.0054$) administration. Binelli et al (5) reported elevated non-esterified fatty acid (NEFA) levels in bGH and bGRF treated cows from this experiment even though they were in positive energy balance. In addition, Liesman et al (28) reported that the activity of hormone sensitive lipase was increased in the adipose tissue of animals in both treatment groups. Finally, adipose tissue depots were depleted in the animals receiving bGH and bGRF treatment. This indicates that the treated animals were undergoing lipolysis and were actually in negative energy balance. Binelli et al (5) explained this discrepancy by proposing that the net energy of lactation (NE_L) for the diet was overestimated which allowed them to overestimate the animals' energy balance. These findings support previously reported observations which have demonstrated that GH inhibits lipogenesis (4, 26, 30, 46, 49) and stimulates lipolysis (1, 4, 13, 26, 31, 50, 51). This ensures that nutrients are directed to the mammary gland rather than being stored as triglycerides in fat tissue. Growth hormone has been shown to enhance the uptake of NEFAs by the mammary gland from blood (29, 33). Adipose tissue mobilization supplies NEFAs to the mammary gland as an additional substrate for milk fat synthesis. As *de novo* synthesis of fatty acids does not appear to be enhanced by either treatment, it is possible that the increased requirement for fatty acids in the milk is met by increased uptake of NEFAs and triglycerides into the mammary gland. Fifty percent of milk fatty acids come from de novo synthesis, 40 % are derived from diet while the remaining 10 % of milk fatty acids come from adipose tissue mobilization (35). The proportion of fatty acids that are derived from adipose tissue mobilization is increased when the animals in negative energy balance. Short and medium chain fatty acids are provided by de novo synthesis while long chain fatty acids are derived from diet and adipose tissue mobilization. Recombinant bGH and bGRF treatment may result in an increase in the extent of fatty acids that are provided by adipose tissue mobilization.

Lipoprotein lipase (LpL) is responsible for hydrolysing triglycerides in very low density lipoproteins (VLDL) and chylomicrons to monoglycerides and fatty acids which can then be taken up passively by the mammary gland. Liesman et al (28) analysed LpL activity in the mammary gland and adipose tissue from the animals examined in this experiment in order to determine the impact of these treatments on uptake of fatty acids from triglycerides. Lipoprotein lipase activity in the mammary gland of treated animals, was found to be unaffected by treatment. This implies that the amounts of fatty acids and monoglycerides taken up by the mammary gland through hydrolysis of triglycerides are also unaltered by treatment.

Conversely, analysis of LpL activity in adipose tissue by Liesman et al (28) revealed that LpL activity was significantly reduced. Our own results in Chapter 4 support the findings of Liesman et al (28). These results combined with the reduced FAS abundance and enhanced lipolysis reported by Binelli et al (5) in adipose tissue of treated animals, support the hypothesis that NEFAs provided the substrates required for the increased milk fat synthesis.

Additional research on the activity of FAS in the mammary gland is required in order to rule out increases in *de novo* synthesis of fatty acids. Our results confirmed previously reported observations for this group of animals and would suggest that *de novo* synthesis is not enhanced in the mammary gland. All milk fat increases are apparently met by the increased uptake of NEFAs into the mammary gland from serum. This supply is met by increased mobilization of adipose tissue as a result of bGH and bGRF treatment.

Conclusion

Fatty acid synthase mRNA abundance in mammary gland was increased by bGH and bGRF treatment while protein abundance was unaltered. The abundance of FAS mRNA and protein in adipose tissue were significantly reduced by both treatments. These collective findings imply that the increased milk fat yield was most likely due to increased uptake of NEFAs. Our results suggest that the increased availability of fatty acids for milk fat does not come from increased de novo synthesis as we had originally hypothesized, but rather was due to increased uptake of NEFAs by the mammary gland as a result of bGH and bGRF stimulated adipose tissue mobilization.

Table 3.1 - Effect of bGH and bGRF on the mRNA Abundance of FAS in Mammary Gland (OD x mm^2)

Treatment	Least Squares Means (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	9.42^a	1.84	n/a	6
bGH	16.55^b	1.84	$p < 0.01$	6
bGRF	15.56^b	1.84	$p < 0.03$	6

* ns $P > 0.10$

** a,b supercripts denote significance

Table 3.2 - Effect of bGH and bGRF on the mRNA Abundance of FAS in Adipose Tissue (OD x mm^2)

Treatment	Least Squares Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	4.22^a	0.73	n/a	4
bGH	0^b	0.63	$p<0.002$	4
bGRF	0^b	0.63	$p<0.002$	4

* a,b superscripts denote significance

Table 3.3 - Effect of bGH and bGRF on FAS Protein Abundance in Mammary Gland (OD x mm^2)

Treatment	Least Squares Means (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	8.81	0.74	n/a	6
bGH	9.29	0.74	ns	6
bGRF	9.48	0.74	ns	6

* ns P>0.10

Table 3.4 - Effect of bGH and bGRF on FAS Protein Abundance in Adipose Tissue (OD x mm^2)

Treatment	Least Square Means (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	6.92^a	1.24	n/a	4
bGH	0.71^b	1.24	$p<0.0062$	4
bGRF	0.55^b	1.24	$p<0.0054$	4

* a,b superscripts denote significance

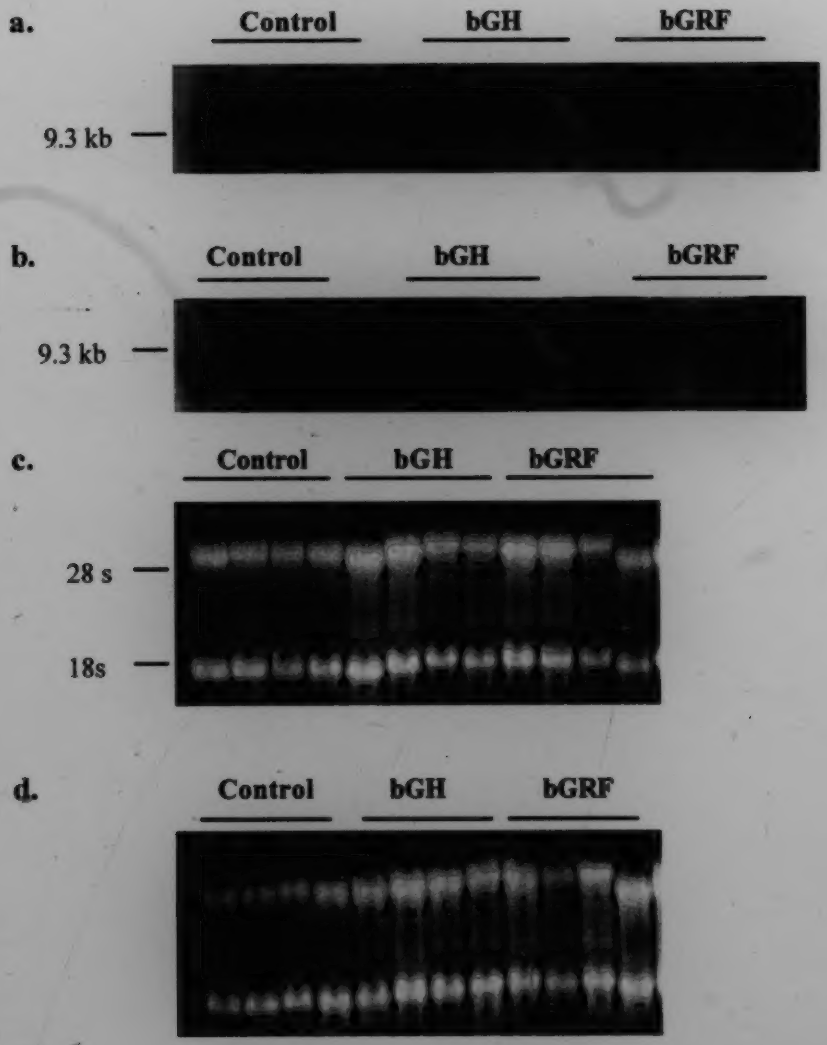
a.
Control
bGH
bGRF
9.3 kb
b.
Control
bGH
bGRF
9.3 kb
c.
Control
bGH
bGRF
28 s
18s
d.
Control
bGH
bGRF

Figure 3.1 - Northern Blot of FAS Message in Mammary Gland

a) Northern blot of FAS mRNA in mammary gland of control, bGH and bGRF treatment groups detected with 2.5 kb rat cDNA probe, animals 1 - 4. b) Northern blot of FAS mRNA in mammary gland of control, bGH and bGRF treatment groups detected with 2.5 kb rat cDNA probe, animals 3 - 6. c) Ethidium bromide stained 18 s rRNA corresponding to a). d) Ethidium bromide stained 18 s rRNA corresponding to b).

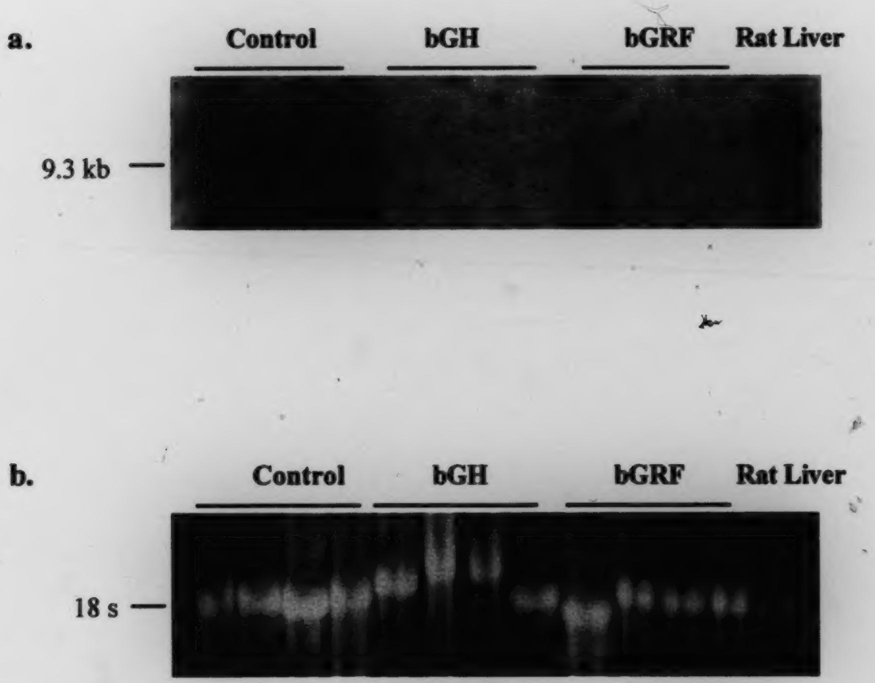
a.
Control
bGH
bGRF
Rat Liver
9.3 kb
b.
Control
bGH
bGRF
Rat Liver
18 s

Figure 3.2 - Northern Blot of FAS Message in Adipose Tissue

a) Northern blot of FAS mRNA in adipose tissue of control, bGH and bGRF treated animals detected with 2.5 kb rat cDNA probe, animals 1 - 4. b) Ethidium bromide stained 18 s rRNA corresponding to a).

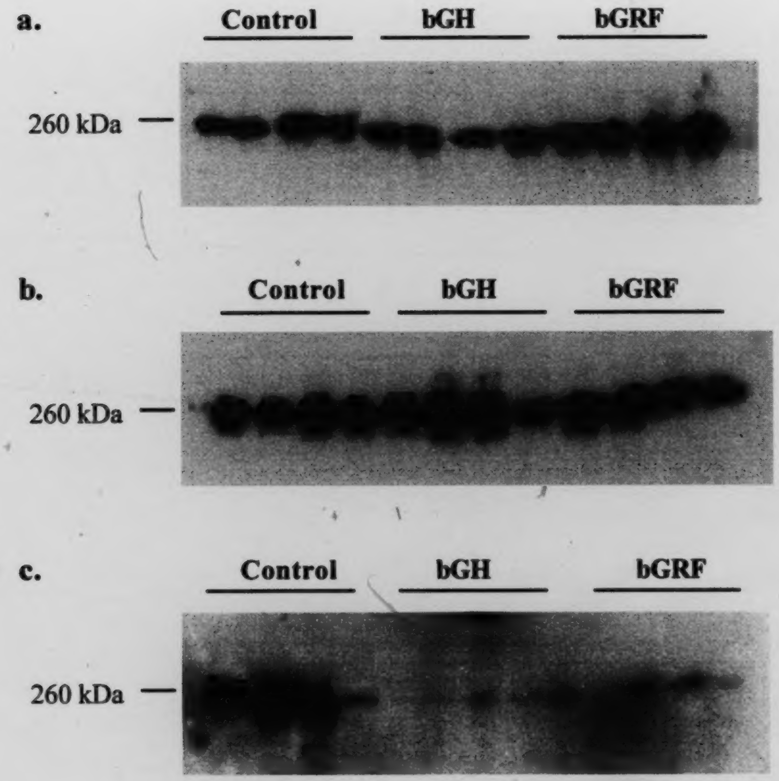
a.
Control
bGH
bGRF
260 kDa
b.
Control
bGH
bGRF
260 kDa
c.
Control
bGH
bGRF
260 kDa

Figure 3.3 - Western Blot of FAS Protein in Mammary Gland and Adipose Tissue

a) Western blot of FAS protein in mammary gland of control, bGH and bGRF treated animals detected with sheep anti-rat FAS/ACL and anti-sheep IgG-horseradish peroxidase, animals 1 - 4. b) Western blot of FAS protein in mammary gland of control, bGH and bGRF treated animals detected with sheep anti-rat FAS/ACL and anti-sheep IgG-horseradish peroxidase, animals 3 - 6. c) Western blot of FAS protein in adipose tissue of control, bGH and bGRF treated animals, animals 1 - 4.

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Chapter 4

Influence of recombinant bovine growth hormone and growth hormone releasing-hormone on the mRNA abundance of lipoprotein lipase and stearoyl-CoA desaturase.

Introduction

At parturition, lactation is induced as prolactin, growth hormone (GH) and insulin-like growth factor I (IGF-I) are increased and progesterone decreases. As a result, the genes involved in milk component synthesis and the uptake of nutrients are induced in the mammary gland. Bovine growth hormone is recognized as the key hormone involved in the maintenance of lactation (5). Development of recombinant DNA technology has enabled low cost production of bGH which enables its use in commercial dairies for the enhancement of milk production. Treatment with recombinant bGH or (bGRF) results in enhanced milk production (2, 3, 6, 12, 14, 15, 20, 21, 26, 28, 29, 31, 49) with little or no change in milk composition (5, 6, 11, 15, 20, 21, 31, 43, 44).

Endogenous GH is released by the anterior pituitary under the inhibitory control of somatostatin and stimulatory effect of bGRF (5). Administration of bGH is believed to stimulate milk production indirectly by elevating insulin like growth factor I (IGF-I) (38, 47) and by partitioning nutrients to the mammary gland from other tissues (2, 4, 33). While mRNA for GH receptors has been isolated from mammary gland (19), the ability of bGH to directly on the mammary gland has not been conclusively demonstrated. Exogenous bGRF acts to increase milk production by stimulating release of endogenous GH from the anterior pituitary. The elevated endogenous GH then acts in the same way as bGH to stimulate IGF-I release and nutrient partitioning.

Lipoprotein lipase (LpL) is produced in mammary gland alveolar epithelial cells of the lactating animals (8, 41) as well as in the parenchymal cells of muscle and adipose tissue. Lipoprotein lipase is then transported to the capillary endothelium where it is bound to the surface and activated (8, 39). Once active, LpL catalyses the hydrolysis of triglycerides contained in very low density lipoproteins (VLDL) and chylomicrons to yield monoglycerides and free fatty acids (32, 37, 39, 40). The products of hydrolysis are then taken up into the tissue via a concentration gradient which is created as the free fatty acids and monoglycerides are utilized by the tissue (40). Lipoprotein lipase is induced in the mammary gland and is reduced in adipose tissue at the onset of lactation to ensure the preferential delivery of nutrients to the mammary gland. The preformed fatty acids taken up in this way are substrate for milk fat synthesis. Lipoprotein lipase is subject to hormonal and control by metabolites (16, 25).

Stearoyl-CoA desaturase (SCD) is normally expressed in the adipose and intestinal tissue of ruminants. At the onset of lactation, expression of the SCD gene is induced at high levels in the alveolar epithelial cells of the mammary gland (10). Stearoyl-CoA desaturase is made up of three proteins, NADH-cytochrome b_5 reductase, cytochrome b_5 and a terminal desaturase, which catalyse the formation of a Δ9 cis double bond in stearic and palmitic acid to yield oleic and palmitoleic acids (10, 22, 34, 35). The double bond formation is enabled by the sequential transfer of electrons from NADH-cytochrome b_5 reductase to cytochrome b_5 to terminal desaturase then to molecular oxygen. The final products are oleoyl-CoA or palmitoleoyl-CoA and water. The terminal desaturase of SCD is subject to hormonal and dietary regulation (34, 35).

The objective of this experiment was to determine the influence of bGH and bGRF administration on the mRNA abundance of LpL and SCD in the mammary gland and adipose tissue. We hypothesized that mRNA abundance of both LpL and SCD would be increased in mammary gland and decreased in adipose tissue when compared to control animals.

Materials and Methods

4.1 - Experimental Design

As described in Chapter 2 and 3, 30 primiparous Holstein cows were assigned to one of ten blocks on the basis of their parturition date. Within each block, one animal was treated with continuous intravenous infusion of 29 mg/day of recombinant bovine growth hormone (Somavubove, The Upjohn Company, Kalamzoo, MI, USA). Another animal was treated with continuous intravenous infusion of 12 mg/day of recombinant bovine growth hormone releasing factor (1-45) homoserine lactone (The Upjohn Company). The last animal in each block received no treatment and served as the control. The bGH and bGRF levels were calculated to produce similar circulating bGH levels based upon a previous experiment by Dahl et al, 1993 (12). Animals received treatment from 118 to 181 ± 1 d postpartum. On the final day of the treatment, animals were slaughtered and tissue was harvested, frozen on liquid nitrogen and stored at - 70 °C.

4.2 - Northern Blotting

4.2.1 - RNA Isolation

Total ribonucleic acid (RNA) was isolated from 200 mg of the mammary gland tissue from six animals of each of the control, bGH and bGRF treatment groups using the TRIzol - chloroform extraction method with an isopropanol precipitation modified from the protocol described by Chomczynski (9). The RNA was resuspended in 100 - 200 µl of sterile Milli-q water. Total RNA was also isolated from four 200 mg aliquots of omental adipose tissue from four animals of each of the treatment groups using the same method. Pellets were resuspended in 25 - 50 µl of sterile Milli-q water, then pooled. The number of animals analysed for each tissue differed as the number of adipose tissue samples was limiting.

4.2.2 - RNA Quantitation and Quality Determination

The RNA was quantified by measuring the absorbance at 260 nm using a Gene Quant spectrophotometer (Pharmacia, Cambridge, UK). Values within 0.1 - 0.8 were considered to be accurate within the linear range of this instrument. Absorbance values were then used to determine the concentration of the RNA in the sample.

The first indication of quality of the RNA was provided by taking the ratio of the absorbance of the sample at 260 nm:280 nm. Values between 1.7 - 2.0 were considered to be of sufficient purity and were used for further analysis. The second determination of RNA quality was provided by running 5 µg of each sample on a 1 % agarose - formaldehyde gel at 100 V for 1.5 h in 1 x MOPS. Samples which had a ratio of approximately 2:1 for the quantity of ethidium bromide stained 28 s:18 s ribosomal RNA species and did not demonstrate any degradation were used for further analysis.

4.2.3 - Detection Level Determination

Ten - 40 µg of total RNA from fresh mammary gland, bovine liver and rat liver were loaded onto a 1 % agarose - formaldehyde gel in order to determine the amount of RNA that was required for detection of the SCD and LpL message. As well, we ran three lanes of polyadenylated RNA enriched samples of 5, 10, and 15 µg along with the other samples. Samples were electrophoresed for 5 h at 100 V with 1 x MOPS. The gel was then placed on a UV transilluminator and the ethidium bromide stained RNA was photographed with Polaroid 665 film. The RNA was transferred to nylon supported nitrocellulose membrane (NitroPure, Micron Separations Inc.) by capillary transfer for 18 - 20 h, and was then fixed to the membrane by baking at 80 °C for 2 h.

4.2.4 - cDNA Probe Preparation for the Dosage Gel

A 2.4 kb cDNA fragment of the human lipoprotein lipase (LpL) coding sequence in pUC19 was obtained from Dr. Tom Clandinin at the University of Alberta and was used to transform *E. coli XLI-blue*. The plasmid-insert DNA was isolated using a plasmid DNA purification kit (Sigma Chemical Company, St. Louis, MO, USA).

A 2.6 kb cDNA fragment of the coding sequence of rat stearoyl-CoA desaturase (SCD) in p91023 - B, was obtained from Dr. Elizabeth Moore at University College Dublin, Ireland. The insert cDNA was excised from the plasmid with EcoRI and was subcloned into pBluescript SK- which was then used to transform *Eschericia coli XLIblue*. The plasmid-insert DNA was isolated using a plasmid DNA purification kit (Sigma Chemical Company).

The LpL insert cDNA was excised from the plasmid using SalI. The SCD insert cDNA was excised from the pBluescript SK- vector using EcoRI. To separate the insert cDNA from the plasmid DNA, the digested sample was run on a 1.2 % agarose gel for 2 h at 50 V in 1 x TAE. The insert cDNA was purified from the agarose using a Gene Clean kit (Bio101, Vista, CA, USA). The LpL and SCD cDNA inserts were labelled with a random primer DNA labelling kit (GibcoBRL / Life Technologies) and $\alpha[^{32}P]$dATP.

4.2.5 - Hybridization, Washing and Autoradiography of the Dosage Gel

The membrane was prehybridized in 25 ml of pre-warmed prehybridization / hybridization solution (6 x SSPE, 0.5 % SDS, 5 x Denhardt's solution) for 1 h at 65 °C. The solution was then replaced with 25 ml of fresh hybridization solution containing the probe reaction and 100 µl of yeast tRNA (GibcoBRL / Life Technologies). Hybridization was carried out at 65 °C for 16 - 18 h. The membrane was washed in three 200 ml changes of low stringency wash (2 x SSPE, 0.1 % SDS) at room temperature. This was followed by one 200 ml high stringency wash (0.1 x SSC, 0.1 % SDS) at 65 °C. The membrane was then placed in an x-ray cassette with two intensifier screens and x-ray film (Kodak X-Omat AR) and exposed to the film for 1 day at - 70 °C. The film was developed and the signals were quantified using an imaging densitometer (BioRad Laboratories, Mississauga, ON).

4.2.6 - Electrophoresis and Transfer of Treated Samples

In order to determine mRNA abundance of LpL in the mammary gland, 30 µg of total RNA from each of the mammary gland samples of six animals from each treatment group were run on duplicate 1 % agarose - formaldehyde gels. Messenger RNA abundance of LpL in adipose tissue was determined by running 20 µg of total RNA from each of the adipose tissue samples from four animals from each of the treatment groups on duplicate 1 % agarose - formaldehyde gels. Gels were run for 5 h at 100 V in 1 x MOPS.

For analysis of the level of expression of the SCD gene in the mammary gland, 10 μg of total RNA from the six animals of each treatment group, were run on duplicate 1 % agarose - formaldehyde gels. Gels were run at 100 V for 5 h in 1 x MOPS. Twenty μg of total RNA from each of the adipose tissue samples were also run on duplicate 1 % agarose - formaldehyde gels using the same conditions of electrophoresis.

Gels were placed on a UV transilluminator and photographed using Polaroid 665 film. The RNA was transferred to nylon supported nitrocellulose membrane (NitroPure, Micron Separations Inc.) by capillary transfer for 18 - 20 h, and was then fixed to the membrane by baking at 80 °C for 2 h.

4.2.7 - Hybridization, Washing and Autoradiography of Treated Samples

Lipoprotein lipase and SCD insert cDNA were labelled with a random primer DNA labelling kit (GibcoBRL/Life Technologies) and α[^{32}P]dATP. Membranes were prehybridized in 30 ml of prewarmed prehybridization / hybridization solution (6 x SSPE, 0.5 % SDS, 5 x Denhardt's solution) for 1 h at 65 °C. Membranes were then hybridized in 30 ml of fresh prewarmed prehybridization / hybridization solution along with the probe reaction and 100 μl of yeast tRNA (GibcoBRL / Life Technologies), for 16 - 18 h at 65 °C.

Membranes were washed in three 200 ml changes of low stringency wash (2 x SSPE, 0.1 % SDS) at room temperature. This was followed by one 200 ml high stringency wash (0.1 x SSC, 0.1 % SDS) at 65 °C. Membranes were then placed in a x-ray cassette with two intensifier screens and x-ray film (Kodak X-Omat AR) and was exposed to the film for 1 - 3 d at - 70 °C. The film was developed and the signals were quantified using an imaging densitometer (BioRad Laboratories, Mississauga, ON). Loading differences were corrected for using quantitation of ethidium bromide stained 18 s rRNA which was then divided from the values of the autoradiograph (7, 35, 37, 45, 51, 52).

4.3 - Statistical Analysis

Statistical significance was determined using the General Linear Model of Least Square Means incorporating treatment, cow and gel variances into the model. The error term was cow(treat). Ydata referred to the data set and was assigned as the dependent variable. As well, Student Neuman-Keuls test was performed. Results for LpL and SCD mRNA abundance were expressed as least square means with SED. Significance was set at $P < 0.10$.

Results

4.4 - Lipoprotein Lipase

4.4.1 - Mammary Gland

Neither bGH nor bGRF administration had any effect on the abundance of the 3.4 and 3.6 kb LpL transcripts in the mammary gland (Figure 4.1). Least square means of OD x mm^2 were 19.01, 20.28 and 12.75 for control, bGH and bGRF treated animals respectively and did not differ significantly (Table 4.1).

4.4.2 - Adipose Tissue

Both bGH ($p < 0.0043$) and bGRF ($p < 0.0119$) reduced the abundance of the 3.4 and 3.6 kb LpL transcripts in adipose tissue significantly (Figure 4.2). Least square means (OD x mm^2) were 18.71, 2.73 and 4.81 for control, bGH and bGRF treated animals respectively (Table 4.2).

4.5 - Stearoyl-CoA Desaturase

4.5.1 - Mammary Gland

Recombinant bGH had a no significant effect on the abundance of the 4.9 kb SCD message in mammary gland (Figure 4.3). Bovine GRF significantly reduced ($p<0.03$) the abundance of the 4.9 SCD transcript. Least square means of arbitrary densitometric units (OD x mm^2) were found to be 11.29, 9.76 and 7.18 for control, bGH and bGRF respectively (Table 4.3).

4.5.2 - Adipose Tissue

The 4.9 kb message of the SCD gene in adipose tissue was reduced below the level of detection by both bGH and bGRF (Figure 4.4) (Table 4.4). There was no difference between the effects exerted by these treatments. The least squares mean mRNA abundance of the control animals was 14.16 OD x mm^2.

Discussion

The galactopoietic effects of bGH and bGRF have been amply demonstrated. In an analysis of milk yield for the animals in this study, Binelli et al (6) reported that there was a significant increase in milk yield ($p < 0.01$) due to bGH or bGRF with no change in milk composition. Specific composition analysis for the milk produced by treated and control animals was not performed, but previous studies by Lynch et al (31) and Apps et al (1) revealed that bGH does not alter fatty acid profiles. In particular, these two studies reported that the concentration of the unsaturated fatty acids palmitoleic (C16:1) and oleic acids (C18:1) were unchanged by bGH treatment. The treated animals were mobilizing body reserves in this experiment which means that the fatty acid composition may have been altered.

As bGH and bGRF increased milk fat yield we assumed that there must be an increase in *de novo* synthesis and uptake of preformed fatty acids from the blood in the mammary gland. This would be necessary to meet the increased substrate requirements for milk fat synthesis. Our previous results (Chapter 2 and 3) suggest that *de novo* synthesis in the mammary gland is not altered by either bGH or bGRF treatment. For this reason, the increased substrate is likely to be provided by uptake of fatty acids from the blood.

Lipoprotein Lipase

The delivery of preformed fatty acids to the mammary gland is accomplished through the action of LpL and by the uptake of serum NEFAs by the mammary gland. Lipoprotein lipase is synthesized in mammary alveolar epithelial cells during lactation and is transported to capillary endothelium where it is bound to the surface and activated. Lipoprotein lipase hydrolyses the triglycerides transported in VLDL and chylomicrons to yield monoglycerides and free fatty acids. These products are then taken up by the mammary gland via a concentration gradient which is created as the mammary gland converts the hydrolytic products back into triglycerides within the tissue. Northern blot analysis of the mRNA abundance of LpL in the mammary gland revealed that there was no significant difference between the treated and control animals. This result was supported by the findings of Liesman et al (30) who reported that the activity of LpL in cultured mammary gland of these experimental animals was unaltered by either treatment. From this we can conclude that the increased substrate did not come from increases in LpL activity in the mammary gland. This is also supported by the results reported in Chapters 2 and 3.

Messenger RNA abundance of LpL in adipose tissue was reduced to 14.6 % and 25.7 % of that of control animals for bGH and bGRF treated animals respectively. Liesman et al reported that LpL activity in omental fat cultures from these animals was also reduced significantly by both treatments (30). The reduced activity of LpL in adipose tissue ensures that triglycerides carried by VLDLs and chylomicrons are preferentially directed to the mammary gland for milk fat synthesis and not to adipose tissue for lipid storage. Control of LpL activity is complex and is determined by control at the transcription and translation level as well as at the point where LpL is transported to the capillary endothelium and is activated. Growth hormone has been previously demonstrated to act on LpL not at the transcription level, but rather at the translation or post-translational level (36). Our results indicate that the effect on adipose tissue by GH is to directly reduce the expression of LpL. Although GH receptors exist in adipose tissue, it is generally accepted that GH exerts its effects on LpL activity and lipogenesis in adipose tissue by inhibiting the effect of insulin (5, 17, 50). Normally, in the absence of elevated GH, insulin stimulates LpL activity in adipose tissue. But when GH is elevated during lactation and further by bGH and bGRF treatment, insulin cannot exert its stimulating effect. Insulin-like growth factor has also been demonstrated to increase LpL activity in the mammary gland (23, 24). Insulin-like growth factor I acts directly on IGF-I receptors in the mammary gland. Vanderkooi et al (47) reported that these animals exhibited elevated IGF-I level following bGH and bGRF treatments. This along with the apparent lack of a increase in *de novo* synthesis of fatty acids in the mammary gland and the constant milk fat percentage, suggested that LpL activity may have been one of the components of milk fat synthesis that was increased.

Binelli et al reported (6) that the animals in this experiment demonstrated significantly ($p < 0.01$) elevated nonesterified fatty acid levels following bGH or bGRF administration. Free fatty acids are believed to negatively affect the concentration of LpL on the surface of capillary endothelium thus negatively affecting LpL activity (36). It is possible that the elevated serum NEFA concentration associated with bGH and bGRF treatment, attenuate the effects of elevated serum IGF-I concentration resulting from bGH and bGRF treatment.

The stable LpL activity and mRNA abundance in the mammary gland support our hypothesis that the increased substrate required to sustain increased milk fat yield, must be provided by increased serum NEFAs. Increased supply of NEFAs is ensured by increased lipolysis and reduced lipogenesis in adipose tissue following bGH and bGRF treatment. Non-esterified fatty acids are taken up into the mammary gland from serum and can the be utilized by the mammary gland for milk fat synthesis. The increased lipolysis which is accompanied by a reduction in LpL mRNA abundance and activity (30) in adipose tissue, ensures that nutrients are supplied preferentially to the mammary gland and not to the other tissues of the body. This is a normal process during lactation that is enhanced by bGH and bGRF treatment.

Stearoyl-CoA Desaturase

The amount of unsaturated fatty acids present in milk fat has been previously demonstrated to be unaffected by bGH treatment (1, 31). Fatty acid composition can be greatly influenced energy balance. The treated animals in this study were apparently in negative energy balance since they were actively undergoing lipolysis. This may have produced differences in the fatty acid composition of the treated animals. Control animals were apparently in positive energy balance. Lynch et al (31) reported that bGH treatment had no effect on the percentage of palmitoleic and oleic acids when compared to controls. However, the control and the treated animals were all in negative energy balance in that study which meant that they were experiencing the same changes in milk composition that arose from adipose tissue mobilization. We had hypothesized that the level of unsaturated fatty acids would not be altered by bGH or bGRF treatment. For this reason, we had expected that the level of desaturase activity would be increased in the mammary gland. To measure the transcription level of SCD in the mammary gland we analysed the mRNA abundance and determined that there was no increase resulting from either bGH and bGRF treatment. In fact, bGRF significantly reduced SCD mRNA abundance ($p < 0.03$). As we do not have the milk fatty acid profile information for the animals in the experiment, these results might indicate that the level of palmitoleic (C16:1) and oleic (C18:1) acids in milk were reduced by the treatments. We can only speculate in the absence of this data.

On average milk fat percentage accounts for 4 % by weight of milk. Of this milk fat, 24 % of fatty acids are C16:0, 2 % are C16:1, 12 % are C18:0, and 24 % are C18:1. If the assumption is correct and the concentration of C16:1 and C18:1 are unaltered by treatment, the increased C16:1 and C18:1 must have been provided by uptake of preformed fatty acids from blood. All fatty acids of C18:0 and above are derived from the blood and come from the diet and adipose tissue mobilization. Unsaturated fatty acids provided by diet in ruminants are low as a result of rumen biohydrogenation of dietary fatty acids. This means that the primary source of unsaturated fatty acids in milk when not produced in the mammary gland, is adipose tissue mobilization. Since SCD activity is normally high in adipose tissue there is some degree of desaturation in lipids stored in adipose tissue in ruminants in spite of dietary deficiency. During lactation this is significantly reduced. Both bGH and bGRF reduced SCD message in adipose tissue still further to below the level of detection. Additional evidence to suggest that the increased substrate for unsaturated fatty acids was not derived from increased uptake of dietary source, is provided by the observation that LpL mRNA and activity are not increased by either treatment. Lipoprotein lipase catalyses the hydrolysis of triglycerides from dietary origin which are carried from the small intestine in chylomicrons. The enzyme is therefore a reflection of the amount of dietary fat that is being taken up by the mammary gland. The amount of dietary fat taken up by the mammary gland was apparently unaffected by either treatment.

The influence of bGH and bGRF on SCD expression in the mammary gland is accompanied by the ability of both hormones to suppress SCD mRNA abundance in adipose tissue below the level of detection. In adipose tissue there is no difference between treatments in the extent of the exerted effect. The absence of detectable SCD message suggests that the activity is also reduced in adipose tissue, although this was not determined. During the dry period, insulin increases the concentration of SCD mRNA as well as activity in adipose tissue (10, 33, 34). Somatotropin suppresses the ability of insulin to stimulate adipose tissue metabolism and in particular desaturation (13). Growth hormone exerts its action on SCD by inhibiting the induction ability of insulin. While this is a normal process of lactation, bGH and bGRF treatment can enhance this process. There has been no evidence presented in literature on the ability of bGH and bGRF to influence SCD directly, but GH is known to act on adipose tissue directly. The reduction in SCD mRNA abundance associated with reduced FAS, ACC and LpL abundance in adipose tissue and elevated NEFA levels in serum (6), ensure enhanced delivery of nutrients to the mammary gland. This enables the composition of milk to remain unaltered by bGH treatment.

Conclusions

Lipoprotein lipase mRNA abundance was unaffected by treatment in the mammary gland, but was reduced to 14 % and 25.7 % of that of control animals for bGH and bGRF respectively, in adipose tissue. Stearoyl-CoA desaturase mRNA abundance was not affected by bGH in the mammary gland, but was reduced by bGRF treatment. Messenger RNA abundance of SCD in adipose tissue was reduced below the level of detection by both treatments. The results suggest that the increased substrate for milk synthesis, is not being met by increased hydrolysis of triglycerides from VLDLs or chylomicrons or from increased desaturation in the mammary gland. Instead, the elevated substrate demand seems to be met by mobilization of fatty acids from adipose tissue depots and from suppressed lipogenic gene expression in adipose tissue.

Table 4.1 - Influence of bGH and bGRF on LpL mRNA Abundance in the Mammary Gland (OD x mm^2)

Treatment	Least Squares Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	19.01	3.44	n/a	6
bGH	20.28	3.44	ns	6
bGRF	12.75	3.44	ns	6

* ns P > 0.10

Table 4.2 - Influence of bGH and bGRF on LpL mRNA Abundance in Adipose Tissue (OD x mm^2)

Treatment	Least Square Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	18.71^a	2.27	n/a	3
bGH	2.73^b	2.27	p<0.0043	3
bGRF	4.81^b	2.27	p<0.0119	3

* ns P > 0.10

** superscript a, b denotes significance

Table 4.3 - Effect of bGH and bGRF on SCD mRNA Abundance in Mammary Gland (OD x mm^2)

Treatment	Least Squares Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	11.29^a	1.20	n/a	6
bGH	9.76^a	1.20	ns	6
bGRF	7.18^b	1.20	p<0.03	6

* ns P>0.10

** superscript a,b denotes significance

Table 4.4 - Effect of bGH and bGRF on SCD mRNA Abundance in Adipose Tissue (OD x mm^2)

Treatment	Least Squares Mean (OD x mm^2)	Standard Error of Difference	Significance	Number of Animals
Control	14.16^a	1.20	n/a	4
bGH	0^b	1.20	p<0.0001	4
bGRF	0^b	1.20	p<0.0001	4

* superscript a,b denotes significance

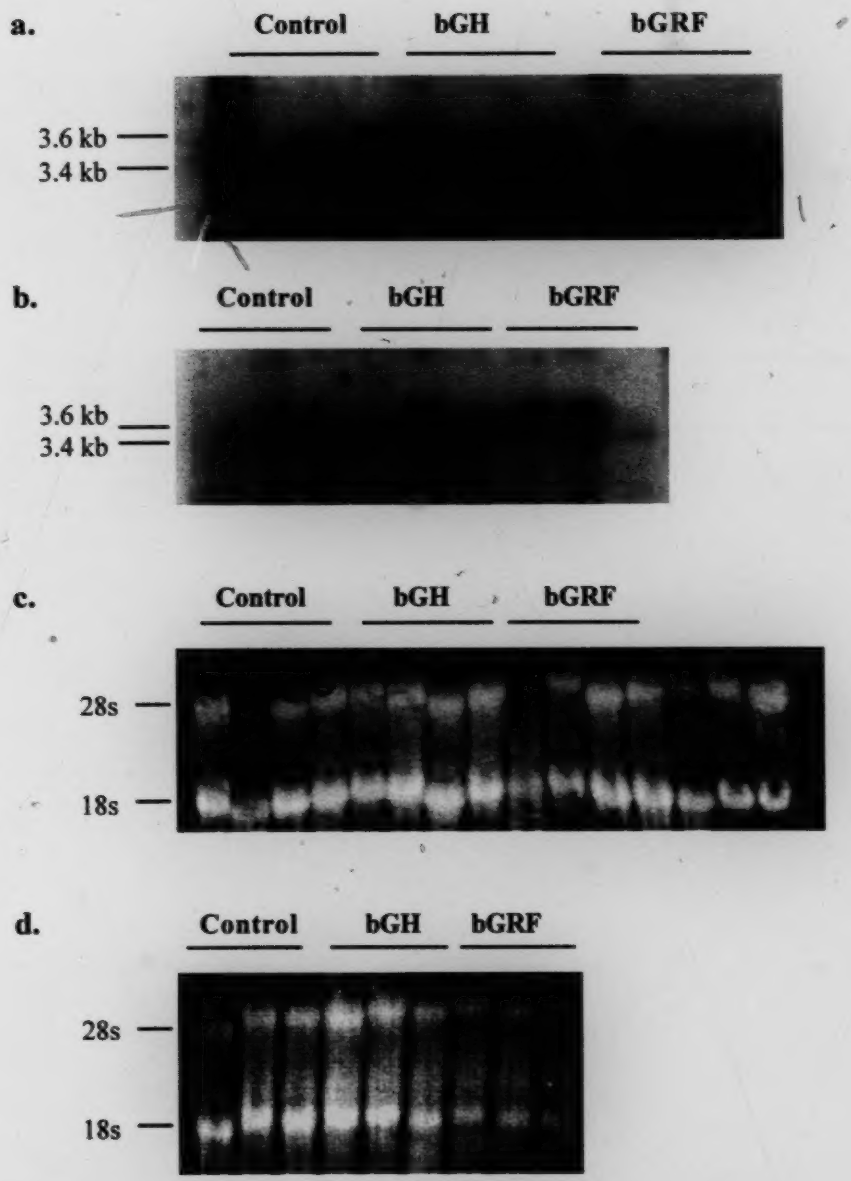
a.
Control
bGH
bGRF
3.6 kb
3.4 kb
b.
Control
bGH
bGRF
3.6 kb
3.4 kb
c.
Control
bGH
bGRF
28s
18s
d.
Control
bGH
bGRF
28s
18s

Figure 4.1 - Northern Blot of LpL Message in Mammary Gland
a) Northern blot of LpL mRNA in mammary gland of control, bGH and bGRF treated animals detected with 2.4 kb human cDNA probe, animals 1 - 4. b) Northern blot of LpL mRNA in mammary gland of control, bGH and bGRF treated animals detected with a 2.4 kb human cDNA probe, animals 2, 5, 6. c) Ethidium bromide stained 18 s rRNA corresponding to a). d) Ethidium bromide stained 18 s rRNA corresponding to b).

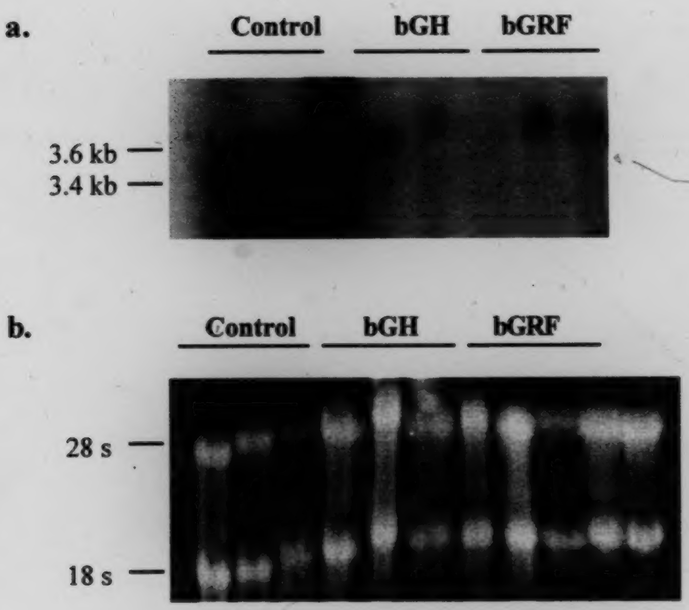
a.
Control
bGH
bGRF
3.6 kb
3.4 kb
b.
Control
bGH
bGRF
28 s
18 s

Figure 4.2 - Northern Blot of LpL Message in Adipose Tissue
a) Northern blot of LpL mRNA in adipose tissue of control, bGH and bGRF treated animals detected with 2.4 kb human cDNA probe, animals 1 - 3. b) Ethidium bromide stained 18 s rRNA corresponding to a).

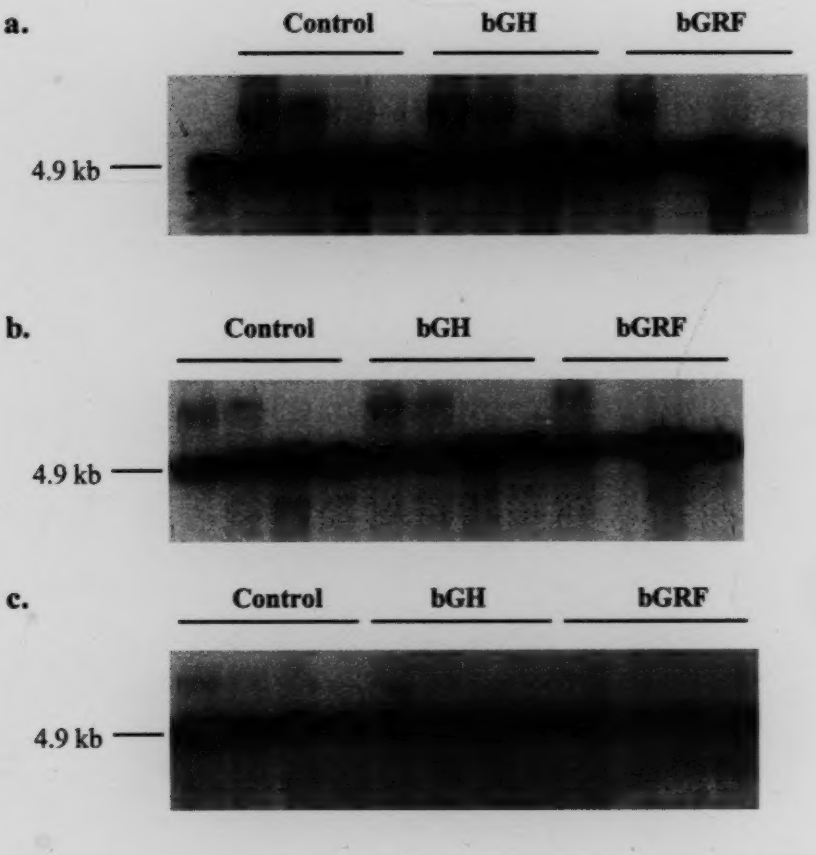
a.
Control
bGH
bGRF
4.9 kb
b.
Control
bGH
bGRF
4.9 kb
c.
Control
bGH
bGRF
4.9 kb

Figure 4.3 - Northern Blot of SCD Message in Mammary Gland
a) Northern blot of SCD mRNA in mammary gland of control, bGH and bGRF treated animals detected with 2.4 kb rat cDNA probe, animals 1 - 4. b) Northern blot of SCD mRNA in mammary gland of control, bGH and bGRF treated animals detected with 2.4 kb rat cDNA probe, animals 1, 2, 5, 6. c) Northern blot of SCD mRNA in mammary gland of control, bGH and bGRF treated animals detected with 2.4 kb rat cDNA probe, animals 3 - 6.

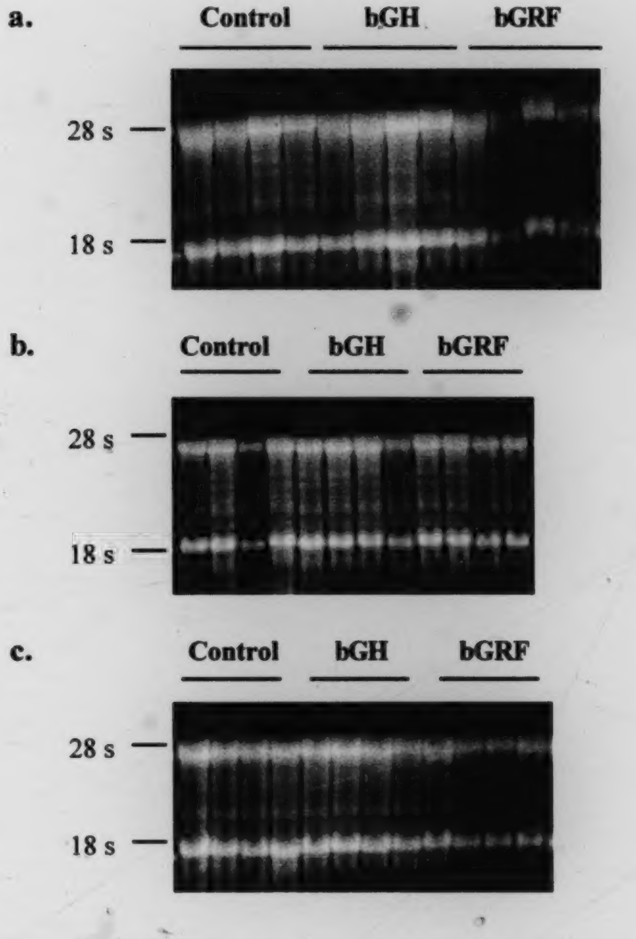
a.
Control
bGH
bGRF
28 s
18 s
b.
Control
bGH
bGRF
28 s
18 s
c.
Control
bGH
bGRF
28 s
18 s

Figure 4.4 - Ethidium Bromide Stained 18 s rRNA in Mammary Gland

a) Ethidium bromide stained 18 s rRNA corresponding to a) in Figure 4.3. b) Ethidium bromide stained 18 s rRNA corresponding to b) in Figure 4.3. c) Ethidium bromide stained 18 s rRNA corresponding to c) in Figure 4.3.

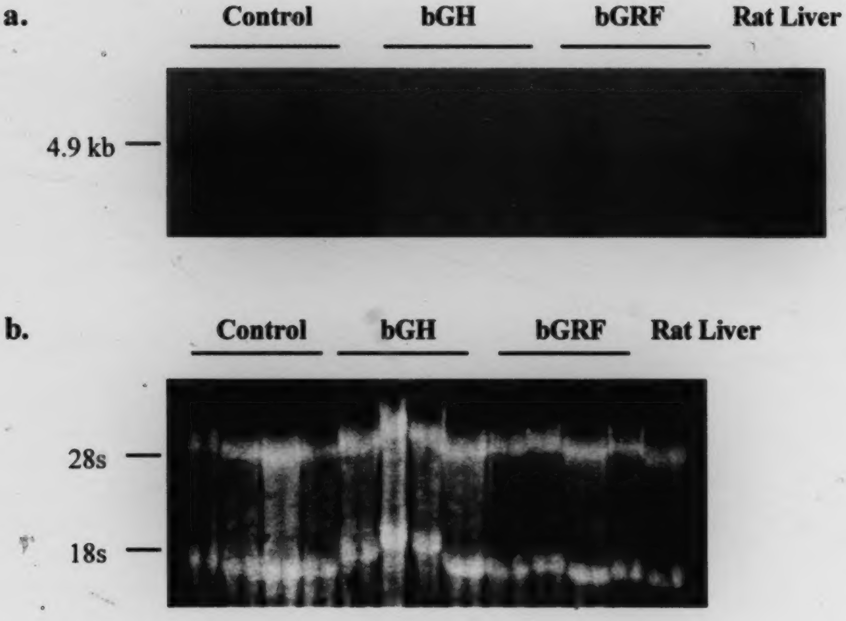
a.
Control
bGH
bGRF
Rat Liver
4.9 kb
b.
Control
bGH
bGRF
Rat Liver
28s
18s

Figure 4.5 - Northern Blot of SCD Message in Adipose Tissue
a) Northern blot of SCD mRNA in adipose tissue of control, bGH and bGRF treated animals detected with 2.4 kb rat cDNA probe, animals 1 - 4. b) Ethidium bromide stained 18 s rRNA corresponding to a).

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Chapter 5 - General Discussion and Conclusions

The Influence of Bovine Growth Hormone and Growth Hormone Releasing Factor on the Synthesis and Uptake of Milk Fatty Acids in Primiparous Holstein Cows.

5.1 - Introduction

Growth hormone (GH) plays an instrumental role in the maintenance of milk production in ruminants. This is accomplished through direct action on tissues such as adipose tissue and liver, and through indirect action on the mammary gland (6). Growth hormone stimulates the release of insulin-like growth factor I (IGF-I) from the liver which is released into the blood stream and transported to the mammary gland where it acts directly (18). Also, GH acts directly on peripheral tissues such as muscle, adipose tissue and liver, to ensure nutrient delivery to the mammary gland.

Administration of bGH and bGRF has been demonstrated to enhance milk production with little or no alteration in the composition of milk. There have also been some reports of milk fat percentage increasing as a result of bGH treatment (10, 12). This requires the nutrient supply to, and metabolic activity in the mammary gland to be increased in order to meet the demand for milk component substrates. Recombinant and pituitary derived bGH reduce insulin responsiveness in adipose tissue so that insulin stimulated lipogenesis is suppressed below control levels (4, 8). In addition, epinephrine stimulated lipolysis is enhanced in treated animals (2, 4). As well, IGF-I levels are elevated which then stimulates the mammary gland (18). These combined forces contribute to increased milk production.

Binelli et al (5) demonstrated that bGH and bGRF treatment resulted in significantly increased milk yield with no change in milk composition in the animals examined in these experiments. Specifically, milk fat concentration was unaltered by either treatment. As a result of the increased milk yield and constant milk fat percentage, there was an increase in the total yield of milk fat in the animals examined. This motivated our analysis of the processes involved in milk fat synthesis.

5.2 - Milk Fat Synthesis

Increased milk fat synthesis can be achieved by increasing the activity of the normal pathways involved in milk fat synthesis as well as by enhancing substrate supply to the mammary gland. In the mammary gland, *de novo* synthesis of fatty acids may be elevated in order to accommodate production of the short and medium chain fatty acids in milk. In addition, LpL activity may be increased to enhance hydrolysis of triglycerides from VLDLs and chylomicrons. This would result in an increase in the supply of preformed monoglycerides and free fatty acids from blood, which are then converted in the mammary gland to triglycerides. Finally, fatty acids for milk fat synthesis can be derived from the uptake of serum NEFAs bound to albumin which are generated primarily by adipose tissue mobilization. In order to more clearly understand these processes we *analysed de novo* synthesis and the uptake of triglycerides into the mammary gland.

Adipose tissue metabolism is altered during lactation to support milk production. This is accomplished by increasing lipolysis and reducing lipogenesis in adipose tissue in order to ensure the preferential supply of nutrients to the mammary gland (3, 7). Lipolysis and reduced lipogenesis are characteristics of animals which are in negative energy balance, i.e. the energy requirements of milk production and maintenance exceed dietary energy. Animals in positive energy balance, i.e. their dietary energy exceeds the energy requirements of milk production and maintenance, do not experience lipolysis, but have suppressed lipogenesis in adipose tissue (4). Enhanced milk production associated with hormones such as bGH can be supported by a modulation of these processes. This results in reduced lipid storage so that lipids are preferentially directed to the mammary gland. Additionally, the increased lipolysis of adipose tissue results in the release of NEFAs into blood when animals are in negative energy balance. These lipids can then be transported to the mammary gland and utilized in milk fat synthesis.

5.2.1 - De Novo Synthesis

To elucidate the status of *de novo* synthesis of fatty acids in the mammary gland, we quantified the mRNA and protein abundance of the key lipogenic enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Acetyl-CoA carboxylase is recognized as the enzyme which catalyses the rate limiting step of fat synthesis and is therefore a good indicator of fatty acid synthesis. Northern blot analysis of the 10.1 kb ACC transcript in the mammary gland, revealed that there was no significant effect of bGH or bGRF treatment on the level of transcription of this gene. In order to determine if there was any influence of either treatment on the abundance of the ACC protein in the mammary gland, Western blot analysis was performed using streptavidin which recognizes the biotin molecule in ACC. Quantitation of the 265 and 280 kDa isozymes revealed that there were no significant effects of either bGH or bGRF treatment on the abundance of this protein.

The second key lipogenic enzyme examined was the multifunctional FAS enzyme which catalyses the sequential addition of two carbons from the malonyl-CoA donor to yield fatty acids of up to 16 carbons (11). Analysis of the 9.3 kb FAS transcript abundance as a measure of gene expression, revealed that bGH increased

FAS expression significantly ($p < 0.01$). Bovine growth hormone releasing factor also significantly ($p < 0.03$) increased the abundance of FAS mRNA in the mammary gland. However, analysis of the abundance of the 260 kDa protein as a measure of translation, revealed that there was no change in the amount of protein in treated animals from either group when compared to control animals.

In adipose tissue, we examined ACC mRNA and protein abundance as an indicator of lipogenic activity. The 10.1 kb ACC transcript was reduced below the level of cDNA probe detection in adipose tissue by both treatments. As well, preliminary Western blot analysis of the 265 kDa isozyme revealed that ACC protein abundance was significantly reduced by both bGH and bGRF treatments.

Autoradiographic analysis was also conducted on the mRNA and protein abundance of FAS in adipose tissue. Messenger RNA abundance of FAS was reduced by both bGH and bGRF treatments to below the level of the cDNA probe detection. As well, bGH and bGRF significantly reduced FAS protein abundance in adipose tissue. Since activity of these enzymes can be influenced both at the level of transcription and translation, these results suggest that lipogenesis is significantly reduced in adipose tissue. This finding is supported by previous experiments which have indicated that GH has the ability to significantly reduce insulin stimulated lipogenesis in adipose tissue (4, 8).

In addition, Liesman et al (13) examined fatty acid synthesis from acetate of tissue cultured mammary gland tissue from these experimental animals as a measure of *de novo* fatty acid synthesis, and found that there was no change in the treated animals when compared to the controls. The increase of milk fatty acids required to support increased milk fat yield must have come either from uptake of dietary fatty acids or from adipose tissue mobilization.

The analysis conducted by Liesman et al (13) on fat synthesis from acetate, revealed that this process was significantly reduced in adipose tissue following treatment with bGH and bGRF. These results support our findings and suggest that fatty acid synthesis is significantly reduced by both treatments. Reduced fat synthesis in adipose would favour nutrient partitioning to the mammary gland to sustain the elevated milk fat yield.

5.2.2 - Uptake of Dietary Lipids

Once we had determined that there was no apparent effect of either treatment on de novo fatty acid synthesis in the mammary gland, we evaluated the influence of treatment on the enzyme involved in the uptake of fatty acids. Lipoprotein lipase catalyses the hydrolysis of triglycerides contained within serum lipoproteins releasing free fatty acids and monoglycerides which can then be taken up by tissues such as mammary gland. Activity of LpL is induced in the mammary gland and is reduced in adipose tissue during lactation (14, 16). Abundance of the 3.4 and 3.6 kb LpL transcript in the mammary gland was not affected by bGH or bGRF treatment. As well, Liesman et al (13) analysed the total activity of LpL in the mammary gland cultures from these animals and determined that bGH and bGRF did not influence the activity of LpL. These results indicate that it is unlikely that the substrate for increased milk fat production is provided by LpL catalysed hydrolysis of triglycerides.

We also examined the influence of bGH and bGRF treatment on the expression of the gene encoding LpL in adipose tissue. This was used as a measure of fatty acid uptake for lipid storage. We observed that bGH and bGRF reduced LpL mRNA abundance to 14.6 and 25.7 % of that of control animals. These results support the work of Liesman et al (13) who analysed the activity of LpL in adipose tissue cultures from these animals and found that both treatments significantly reduced its activity. This suggests that the uptake of free fatty acids into adipose tissue mediated by LpL catalysed hydrolysis of triglycerides, is diminished by both of these treatments. This process ensures that triglycerides contained within serum lipoproteins are preferentially directed to the mammary gland in order to support the treatment enhanced milk fat production.

5.2.3 - Adipose Tissue Mobilization

Animals in this trial were in early-mid lactation and were calculated to be in positive energy balance (5). While the energy balance of treated animals was lower than that of the control animals, treated animals were also calculated to be in positive energy balance. However, bGH and bGRF treatment significantly increased NEFA concentration. Elevation of NEFAs is a direct indication of adipose tissue mobilization resulting from lipolysis which is a characteristic of negative energy balance. Binelli et al (5) explained this result by stating that the calculated net energy of lactation (NE_L) for the diet must have been overestimated and the animals were in negative energy balance. This is further supported by the findings of Liesman et al (13) which demonstrated that these animals also had elevated hormone sensitive lipase in the adipose tissue following bGH and bGRF treatment. As well, Binelli et al (5) reported that the carcass fat depots were diminished by both treatments. Finally, dry matter intake (DMI) of the treated animals was not increased in spite of the enhanced milk production (5) which would force the animal to mobilize body reserves in order to meet the substrate demand for increased milk synthesis. All of these factors support the observation that the animals were mobilizing body reserves to satisfy the energy requirements of milk production. The animals were therefore in negative energy balance following bGH and bGRF treatment.

Milk fat is derived from three sources: *de novo* synthesis in the mammary gland, uptake of dietary lipids and uptake of fatty acids provided by adipose tissue mobilization. Normally *de novo* synthesis is estimated to provide 50 % of milk fatty acids, diet is the source of another 40 %, while the remaining 10 % are provided by adipose tissue mobilization (17). During negative energy balance, the percentage of fatty acids which are derived from adipose tissue mobilization is increased. This is the case with the treated animals in this experiment. Also in these treated animals, *de novo* fatty acid synthesis appears not to be increased by either bGH or bGRF treatment based on the lack of response in ACC and FAS mRNA and protein abundance, and the lack of increase in fat synthesis from acetate (13). Furthermore, uptake of dietary triglycerides is apparently not increased by either treatment since neither the mRNA abundance nor the activity (13) of LpL are altered by either treatment. Thus, the supply of free fatty acids and monoglycerides derived from the hydrolysis of triglycerides contained within lipoproteins would not be altered by treatment. Finally, serum NEFA concentration was enhanced indicating that adipose tissue mobilization is increased by both treatments. Free fatty acids mobilized from adipose tissue could then be transported to the mammary gland in the blood and taken up directly for fat incorporation into milk fat. Thus, NEFAs may be the primary source of the fatty acids required for additional milk fat production in treated animals. The lack of effect on the enzymes responsible for *de novo* synthesis provide additional support for the hypothesis that NEFAs are the primary source of the enhanced milk fat production.

5.2.4 - Fatty Acid Desaturation

We were also interested in the influence of treatments on the action of SCD in the mammary gland. Previous experiments have demonstrated that bGH has no effect on the amount of unsaturated fatty acids in milk and in particular palmitoleic

(C16:1) and oleic (C18:1) acids (1, 15). In order to accommodate the increased demand for unsaturated fatty acids required to keep their concentration in milk constant, there must either be increased desaturation of fatty acids in the mammary gland by SCD or increased uptake of C16:1 and C18:1 from blood. We analysed the mRNA abundance of SCD as a measure of the effect of bGH and bGRF on endogenous desaturation in the mammary gland. The results demonstrated that bGH had no effect on SCD mRNA abundance in the mammary gland, but bGRF negatively ($p < 0.03$) affected SCD expression. Since control can be exerted at the level of transcription and may determine SCD total activity, the results obtained suggest that SCD activity is unaltered by bGH treatment and slightly diminished by bGRF. As a result, the increased unsaturated fatty acid substrate required to maintain a constant level of unsaturation in milk, must be met by uptake of fatty acids from blood. In this case, fatty acid profiles were not analysed, however, previous experiments by Apps (1) and Lynch (15) support our hypothesis that these are not altered by treatment. The increased concentration of fatty acids may be met by uptake of long chain unsaturated fatty acids from blood.

5.3 - Conclusions

The results obtained in this series of experiments suggest that the increased substrate for milk fat synthesis that is required following bGH and bGRF treatment, is provided by increased uptake of NEFAs by the mammary gland. We had hypothesized that bGH and bGRF treatment would result in an increase in the expression of ACC, FAS, LpL and SCD in the mammary gland. We had also proposed that the expression of these genes in adipose tissue would be downregulated by these treatments. Our hypothesis about mammary gland lipogenic status was not supported as we did not see any significant changes in the genes/enzymes of interest. Our hypothesis in regards to adipose tissue was supported by our experimental data as we saw significant decreases in the mRNA abundance of all the genes of interest.

We had hypothesized that we would see increases in the lipogenic function of the mammary gland to support increased milk fat yield associated with bGH and bGRF treatment. Our data indicate that *de novo* synthesis and uptake of fatty acids and monoglycerides from triglyceride containing lipoproteins were not enhanced by treatment, implying that these processes are not enhanced in the mammary gland. For this reason, it is reasonable to assume that the third source of milk fatty acids - adipose tissue mobilization, accounted for the observed differences in milk fatty acid yield. This is supported by the fact that the animals receiving the bGH and bGRF treatments demonstrated elevated NEFA concentration when compared to the control animals (5). As well, carcass energy and size of fat depots were reduced in the treated animals (5), demonstrating that energy was being mobilized to support the increased substrate required by the mammary gland. Finally, the reduced lipogenic activity in adipose tissue rendered more substrate available to the mammary gland to favour milk fat synthesis.

As nutrient supply to the mammary gland would appear to be increase due to a reduction in adipose tissue lipogenic activity, enhanced lipogenic activity and uptake of triglycerides in the mammary gland would be expected. However, as we have seen this was not the case. In the case of ACC and FAS, the apparent inability to increase *de novo* synthesis is surprising since the downregulation of these genes and reduced fat synthesis from acetate in adipose tissue, would potentially provide more substrate to the mammary gland for *de novo* synthesis. Since increased substrate normally results in an increase in the activity of ACC and FAS, we would expect that the elevated substrate would lead to increased lipogenic activity. It is possible that the enhanced delivery of substrate leads to only marginal changes in the mRNA and protein abundance which are not quantifiable. Lipoprotein lipase can be downregulated by elevated NEFA concentration (9) which may ~~actually~~ attenuate the stimulatory effect of the treatments on lipoprotein activity. The increased circulating NEFAs appear to have been the primary substrate for enhanced milk fat yield.

Recombinant bGH and bGRF treatments are likely to increase the total yield of unsaturated fatty acids. Previous observations have indicated that the percentage of oleic and palmitoleic acids are not altered following bGH treatment (1, 15). We observed that the mRNA abundance of SCD is not altered by either treatment which suggests that the level of de novo desaturation in the mammary gland is not enhanced. If the reports of unchanged fatty acid composition are supported in this experiment also, we can assume that the increased substrate may be derived from the uptake of long chain NEFA which are unsaturated and the uptake of these same fatty acids through the action of LpL on lipoproteins.

Additionally, our results indicate the status of the animals following 63 days of bGH and bGRF treatment. At this time, animals had already adjusted to the treatment by reducing adipose tissue reserves. In the early days of treatment, it is possible that the effects on mammary gland ACC, FAS, LpL and SCD may have been more profound. Furthermore, if the experiment had been continued for a longer period of time so that the animals were in positive energy balance, it is likely that the results would be modulated by the change in energy status of the animal.

5.4 - Implications

The results obtained in this body of work represent the first direct measure of the action of bGH and bGRF on the gene expression and protein synthesis of ACC, FAS, LpL and SCD in cattle. Our results reveal that there is little direct impact of either treatment on the mammary gland, but that there are profound effects on adipose tissue. The net result is that substrate is preferentially directed to the mammary gland from the body energy stores to support milk fat synthesis.

The information that was obtained in this experiment brings us closer to the ultimate goal of manipulating the percentage and composition of milk fat. Consumers are demanding a lighter product which has a lower milk fat percentage and a higher proportion of long chain unsaturated fatty acids. These forces drive the research in this area as we attempt to decrease milk fat and increase the concentration of unsaturated fatty acids. Understanding the processes involved in milk fat synthesis and its regulation will enhance our ability to manipulate the composition of dairy products. This will enable the dairy industry to respond more effectively to changing consumer demand.

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Appendix 1 - Methodology

I. DNA Probe Preparation

When plasmids containing the cDNA insert are first received from the source they must be amplified in order to increase the volume of the working stock. A bacterial culture is transformed with the plasmid which is then grown under antibiotic selective conditions in order to select for the plasmid containing colonies. The plasmid is then purified from the bacteria in preparation for its use and is stored at -20 °C. Plasmid preparation was required for all of the Northern blotting experiments reported in Chapters 2 - 4. After the plasmid DNA is isolated, the insert cDNA must be isolated from the plasmid DNA in preparation for Northern hybridization analysis.

1.1 - Transformation of Bacteria with Plasmid Containing Insert

Solutions for Plasmid Amplification

LB Liquid Medium:
To make up 500 ml:
To 450 ml of deionized water add:

bacto-tryptone	5.0 g
bacto-yeast	2.5 g
NaCl	5.0g

Use a magnetic stirrer until the solids have dissolved then adjust the pH to 7.0 with 5 N NaOH. Adjust the volume to 500 ml and autoclave.

LB-agar-ampicillin plates:
Make up 1 L of LB and add 15 g of bacto-agar. Autoclave. After autoclaving, let the solution cool to 65 °C then add ampicillin stock solution (50 mg/ml) to the LB-agar to a final concentration of 50 μg/ml. Pour the plates carefully. This volume allows 20 - 24 plates to be poured. These plates can be stored for up to 30 days at 4 °C.

Ampicillin stock:
Dissolve 50 mg of ampicillin in 1 ml of water.

1. Thaw competent cells (i.e. *Eschericia coli XLI blue*) on ice for 15 min.

2. Add 1 µl of plasmid suspension (i.e. ACC in pGEM7zf+) to 100 µl of competent cells in a 6 ml Falcon conical tube.

3. Incubate on ice for 45 min.

4. Heat shock the competent cells by incubating at 42 °C for 45 s.

5. Incubate the cells on ice for 2 min.

6. Add 100 µl of LB medium to the cells.

7. Incubate the cells and medium in a 37 °C shaker incubator for 1 h at 200 rpm.

8. Plate the cells on an LB-agar plate containing ampicillin at a concentration of 50 µg/ml.

9. Incubate for 16 - 18 h at 37 °C.

10. Wrap the plate in parafilm then store at 4 °C.

11. Maintain the colony by re-plating on LB-agar-ampicillin plates every 30 d.

1.2 - Plasmid DNA Isolation

Plasmid DNA was isolated from the bacteria using a plasmid purification kit (Sigma Chemical Company, St. Louis, MO, USA). The plasmid DNA containing the insert cDNA was eluted using 50 µl of sterile water. The DNA was then stored at - 20 °C.

1.3 - Purification of Insert DNA

Solutions for Insert cDNA Isolation

50 x TAE

To make 500 ml:

121 g Tris base
28.6 ml glacial acetic acid
50 ml 0.5 M EDTA, pH 8.0

Make up to 500 ml with sterile milli-q water and autoclave.

Orange G Buffer

In a volume of 6.5 ml:

4.35 ml 30 % Ficoll
1.0 ml 2 % Orange G solution
0.65 ml 0.5 M EDTA, pH 7.0
0.5 ml water

Aliquot into 500 µl aliquots. Freeze at - 20 °C for long term storage. Keep working stock at room temperature.

1. Incubate the plasmid containing the insert in the appropriate restriction enzyme with 3 units of enzyme for each µg of DNA, for 1.5 h at 37 °C

2. Prepare a 1.2 % agarose gel for separating the insert DNA from the plasmid DNA. The gel is prepared by combining: 0.36 g agarose, 30.0 ml 1 x TAE, and 15 µl 1.0 mg / ml ethidium bromide. The agarose is then dissolved in 1 x TAE by heating for 1 minute in a microwave. The solution is cooled to 55 °C and the ethidium bromide is added. The gel is then poured in a small gel submarine with a 8 well comb and is allowed to solidify for 20 minutes before the samples are added.

3. Samples are prepared by combining the digested plasmid solution with 5 µl of Orange G buffer. 10 µl of undigested plasmid is also combined with 5 µl of Orange G buffer. 1 µl of DNA Molecular Weight Marker II (125 - 23130 bp, Boehringer Manheim) is added to 10 µl of water and 5 µl of Orange G buffer.

4. Load samples onto the gel which has been submerged in 1 x TAE.

5. Run the gel at 50 V for 2 h.

6. Visualize the DNA on a UV transilluminator and photograph using Polaroid 665 film.

7. Excise the insert band from the gel using a sterile scalpel blade and place into a pre-weighed 1.5 ml Eppendorf tube. Re-weigh the tube and the gel slice.

8. Elute the DNA from the gel with a GeneClean Kit (Bio101, Vista, CA, USA) using the following procedure:
   - Add 3 volumes of sodium iodide to the gel slice.
   - Heat at 45 - 55 °C for 10 min to melt the agarose.
   - Add 5 µl glass milk solution and incubate on ice for 5 min.
   - Centrifuge 5 s. Discard the supernatant.
   - Wash the pellet three times with 200 - 500 µl of New Wash. Centrifuge 5 s between each wash. Remove all of the New Wash on the last step.
   - Resuspend the pellet in 12 µl of sterile milli-q water.
   - Heat at 45 - 55 °C for 10 min to elute the DNA from the glass milk.
   - Centrifuge 30 s and transfer the supernatant to a fresh tube.
   - Discard the pellet.

9. Store isolated insert DNA at 4 °C or at -20 °C for long term storage.

1.4 - Subcloning Insert DNA into a Different Vector

1. Purify insert DNA as described above.

2. Linearize the vector to be used for subcloning using the same restriction enzymes as were used to excise the insert DNA from the previous vector.

3. Using a 1 % agarose gel, separate the linearized DNA from the non-linearized DNA. Excise the band of linearized DNA from the gel. Elute the DNA from the gel using the same procedure as described above.

4. Carry out a ligation reaction. The ligation mixture contains: 2 µl 10 x ligase buffer, 1 µl T4 ligase, 3 µl linearized vector DNA, 10 µl insert DNA and 4 µl water.

5. Let the solution equilibrate at 37 °C for 15 min.

6. Incubate overnight at 15 °C.

7. Transform competent cells as described above with 5 µl of ligation mixture.

1.5 - Detection of Subcloned Plasmid Containing Colonies

1. Prepare LB-agar plates containing 50 µg/ml ampicillin as described above.

2. Add 50 µl of 10 mg/ml X-gal (GibcoBRL / Life Technologies) and incubate at 37 °C for 1 h.

3. Add 4 µl 0.5 M IPTG to the transformed competent cells.

4. Plate cells on the LB-amp plates and incubate overnight at 37 °C.

5. Observe plate in the morning. Blue colonies are positive for the subcloned plasmid.

6. Prepare miniprep with the positive colonies by inoculating each miniprep with an individual positive colony. Mini-preps and plasmid isolation are performed as above.

7. Following plasmid isolation, confirm subcloning by using the restriction enzymes to excise the insert DNA.

8. Run the digested plasmid on a 1 % agarose gel to confirm the presence of the insert DNA.

9. Store isolated subcloned plasmid at 4 °C.

2. Northern Blotting

Northern blotting is used to determine the state of transcription of the gene of interest. Ribonucleic acid is isolated from the tissue and is separated using an electrical current on the basis of its size. The RNA is then transferred to a membrane which may be nylon or nitrocellulose in composition. The level of transcription of the gene being examined is measured by hybridizing a radiolabelled cDNA probe corresponding to that gene's sequence under high stringency conditions. The resultant image that is produced by exposing x-ray film to the radiolabelled products of hybridization, reveals the level of expression of the gene which can then be quantified. This technique is employed in Chapters 2 - 4.

2.1 - RNA Isolation

Solutions for RNA Isolation

- TRIzol (GibcoBRL / Life Technologies)
- chloroform (Fisher Scientific)
- isopropanol (Sigma Chemical Company)
- 75 % ethanol

2.1.1 - Mammary Gland, Rat Liver and Bovine Liver

1. Grind 200 mg of tissue in a mortar and pestle which had been cooled overnight at - 80 °C.

2. Homogenize the tissue in 2 ml of TRIzol reagent (Gibco BRL / Life Technologies, Grand Island, NY, USA) using a Polytron power homogenizer at maximum speed for 30 s in a 14 ml round bottomed Falcon tube (Becton Dickinson, Franklin Lakes, NJ, USA).

3. Incubate samples at room temperature for 5 min.

4. Centrifuge at 9500 rpm (< 12 000 x g) at 4 °C for 10 min in a Beckman JA-120.1 rotor (Beckman Instruments Ltd., Palo Alto, USA) (all subsequent centrifugation steps are also carried out in this rotor).

5. Transfer the supernatant to a fresh 14 ml Falcon round bottomed tube.

6. Add 0.4 ml chloroform to the supernatant. Shake vigorously for 30 s. Incubate for 3 min at room temperature.

7. Centrifuge at 9500 rpm (< 12 000 x g) for 15 min at 4 °C.

8. Using a pasteur pipette, transfer the upper aqueous layer to a fresh 14 ml Falcon conical tube.

9. Add 1 ml isopropanol to precipitate the RNA. Vortex then incubate at room temperature for 1 - 1.5 h.

10. Centrifuge at 9500 rpm (< 12 000 x g) for 10 min at 4 °C.

11. Discard the supernatant. Wash the pellet in two 2 ml washes of 75 % ethanol. Centrifuge at 7500 rpm (7500 x g) at 4 °C for 5 min. On the last wash, carefully remove the ethanol using a pipette attached to a vacuum trap.

12. Resuspend the pellet in 100 - 200 µl of sterile milli-q water depending upon the size of the pellet.

13. Warm the RNA suspension in a 55 - 65 °C water bath for 10 min. Centrifuge momentarily to collect the sample at the bottom of the tube.

14. Transfer the resuspended RNA to a 1.5 ml Eppendorf (Brinkman Instruments Inc., Westbury, NY, USA).

15. Store the samples at -80°C.

2.1.2 - Adipose Tissue

1. Grind 200 mg of adipose tissue x 4 in a pre-chilled mortar and pestle.

2. Homogenize the tissue in 4 ml TRIzol with a Polytron power homogenizer at maximum speed for 30 s in a 14 ml Falcon conical tube.

3. Incubate at room temperature for 5 min.

4. Centrifuge at 9500 rpm (< 12 000 x g) for 10 min at 4 °C.

5. Transfer supernatant to a fresh 14 ml Falcon round bottomed tube.

6. Add 0.8 ml chloroform to supernatant. Shake vigorously for 30 s. Incubate at room temperature for 3 min.

7. Centrifuge at 9500 rpm (< 12 000 x g) for 15 min at 4 °C.

8. Using a pasteur pipette, transfer the upper aqueous layer to a fresh Falcon tube.

9. Add 3.0 ml isopropanol, vortex and incubate at room temperature for 1.5 h.

10. Centrifuge for 10 min at 9500 rpm (< 12 000 x g) at 4 °C.

11. Wash pellet in 2 changes of 1 ml of 75 % ethanol. Centrifuge for 5 min at 7500 rpm (7500 x g) at 4 °C. Remove the ethanol at the end of the last wash using a pasteur pipette attached to a vacuum line.

12. Resuspend the pellet in 25 - 50 µl based upon the size of the pellet.

13. Incubate in a 55 - 65 °C water bath for 10 min.

14. Centrifuge momentarily to collect the sample in the bottom of the tube.

15. Transfer the sample to a 1.5 ml Eppendorf tube and freeze the sample at - 80 °C.

2.2 - RNA Quantitation and Quality Determination

2.2.1 - Quantitation

1. Thaw the RNA samples on ice. Vortex gently to ensure even distribution of the RNA.

2. Add 5 µl of the sample to 995 µl of sterile milli-q water (1/200 dilution).

3. Place the entire sample into a 1 ml cuvette and measure the absorbance of the sample at 260 nm.

4. Apply the following formula to the absorbance value to determine the concentration of the sample:

   * The conversion factor for RNA is 0.04 μg/μl per 260 nm OD unit
   OD_{260} x dilution factor x conversion factor = μg RNA / μl
   e.g. 0.8 x 200 x 0.04 = 6.4 μg / μl

2.2.2 - Quality Determination

1. Prepare sample as above.

2. Measure the absorbance of the sample at 260 and 280 nm.

3. Take the ratio of 260/280 nm.

4. Keep samples which are within the range of 1.7 - 2.0.

5. Aliquot 5 μg of sample from samples which are within this range.

6. Speed vacuum the samples down to 2 - 3 μl if necessary.

7. Add 10 μl of RNA sample dye.

8. Heat sample at 70 °C for 10 min.

9. Prepare a 1 % mini-gel by combining: 0.25 g agarose, 21 ml sterile milli-q water, 1.35 ml formaldehyde and 2.5 ml 10 x MOPS.

10. Run the samples on the gel for 1 h at 100 V with 1 x MOPS.

11. Place the gel on a UV transilluminator and photograph the gel using Polaroid 665 film.

12. Compare the relative abundance of the 28 s to 18 s ribosomal RNA.

13. Samples which have a ratio of 2:1 for 28 s: 18 s ribosomal RNA are then preserved for further analysis.

2.3 - RNA Gel Electrophoresis and Transfer

Solutions for Electrophoresis and Transfer

10 x MOPS

To make 1 L:
41.86 g of MOPS
6.80 g sodium acetate trihydrate
3.72 g sodium EDTA

Dissolve in 950 ml water. Adjust pH to 7.0 with 10 N NaOH. Adjust the volume to 1 L then filter sterilize using a filter of 0.45 microns in size. Store in a light proof container in a dark cupboard.

20 x SSC

To make 1 L:
175.3 g NaCl
88.2 g trisodium citrate

Adjust the pH to 7.0 with a few drops of HCl. Autoclave to sterilize solution.

Loading Dye for RNA gel electrophoresis:

175 µl sterile deionized water
25 µl 10 mg/ml ethidium bromide
80 µl glycerol
160 µl 10 x MOPS
80 µl saturated bromophenol blue solution

Sample Dye for RNA gel electrophoresis

173 µl formaldehyde
347 µl loading dye
480 µl deionized formamide

2.3.1 - Electrophoresis

1. Prepare a 1 % agarose-formaldehyde denaturing gel by combining: 1.4 g agarose, 120 ml sterile milli-q water, 14 ml 1 x MOPS and 7.6 ml formaldehyde.

2. Aliquot a volume of RNA appropriate to the mass (µg) of RNA required for detection of the message of the gene of interest.

3. Dry the samples down to approximately 3 µl in a speed vacuum (Hetovac).

4. Add 10 µl of sample dye.

5. Heat the samples in a water bath at 70 °C for 10 min. Immediately quench the sample on ice.

6. Centrifuge momentarily to collect the sample in the bottom of the tube.

7. Load the sample into the solidified gel covered by 1 x MOPS.

8. Separate RNA by running the samples on the gel using 1 x MOPS as the buffer at 100 V for 5 h. Buffer is circulated constantly using a capillary pump in order to maintain a constant ion concentration.

9. Following electrophoresis, visualize the RNA on a UV transilluminator.

10. Photograph the gel using Polaroid 665 film (Polaroid Corporation, Cambridge, MA, USA).

11. Cut the gel above the wells and below the smallest visible RNA species.

12. Wash the gel in two changes of 10 x SSC for 10 min each.

13. While the gel is being washed, cut a piece of NitroPure membrane (Micron Separations Inc.) which is the same size as the gel and soak it in water for 10 min. Cut nicks on the membrane in order to maintain the orientation of the membrane.

14. Cut three pieces of 3 mm Whatman chromatography paper to the same size of the gel and prepare them by soaking them in 10 x SSC.

2.3.2 - RNA Transfer

1. Fill the bottom of a large, baked pyrex dish with 10 x SSC.

2. Place two petri dishes on top of each other in the centre of the baked dish.

3. Place a glass plate on top of the petri dishes.

4. Place a piece of 3 mm Whatman chromatography paper on top of the glass plate. The filter paper should be the width of the glass plate and long enough so that the ends of the wick are submerged in the 10 x SSC.

5. Saturate the wick with 10 x SSC and roll out air bubbles between the filter paper and the glass plate using a 10 ml glass pipette.

6. Following the washing of the gel, carefully place the gel face down onto the wick.

7. Cover the gel with 10 x SSC and roll out air bubbles.

8. Place the pre-soaked membrane face down on the gel. Cover with 10 x SSC and roll out air bubbles.

9. Cover the membrane with the three pieces of filter paper being careful to roll out the air bubbles after the addition of each piece.

10. Cover the area of the wick not covered by the gel with pieces of parafilm in order to ensure the transfer of solution occurs only in the area covered by the gel.

11. Place a stack of paper towels about 10 cm in height on top of the filter papers.

12. Place a glass plate on top of the paper towels.

13. Place a weight on top of the glass plate and ensure that the top of the apparatus is level.

14. Use plastic wrap to cover the area of the glass dish not covered by the glass plate in order to prevent the evaporation of the 10 x SSC.

15. Let the transfer proceed for 18 - 20 h.

16. Place the gel and the membrane on the UV transilluminator in order to determine if all of the RNA was transferred from the gel to the membrane.

17. If there is no RNA remaining on the gel, bake the membrane at 80 °C for 2 h.

18. Store the membrane at 4 °C.

2.4 - Northern Hybridization

2.4.1 - Probe Labelling

1. Combine 5 µl of purified insert cDNA with 18 µl sterile milli-q water.

2. Heat in a boiling water bath for 5 min. Place directly on ice.

3. Centrifuge momentarily to collect the sample in the bottom of the tube.

Perform the following additions on ice:

4. Add 2 µl each of dCTP, dGTP and dTTP from the Random Primer Labelling kit (Gibco BRL / Life Technologies, Grand Island, NY, USA).

5. Add 5 µl of $\alpha[^{32}P]$dATP. Mix by flicking.

6. Add 1 µl of Klenow fragment.

7. Incubate at room temperature for 4 h.

8. Add 5 µl stop buffer.

9. Prepare probe for hybridization by adding 100 µl of yeast tRNA (10 mg/ml) (Gibco BRL / Life Technologies). Heat probe in boiling water bath for 2 min then store on ice until addition to hybridization solution.

2.4.2 - Prehybridization and Hybridization of Membranes

Solutions for Prehybridization and Hybridization

20 x SSPE

3 M NaCl, 0.2 M monobasic sodium phosphate, 0.2 M sodium EDTA.
To make 1 L:
175.3 g NaCl
27.6 g NaH_2PO_4-H_20
7.4 g EDTA

Dissolve in 900 ml. Adjust pH to 7.4 with 10 N NaOH. Adjust volume to 1 L. Autoclave.

50 x Denhardt's Solution

1% Ficoll
1% PVP
1% Fraction V BSA

Filter sterilize. Store in 10 ml aliquots and freeze at -20 °C.

Hybridization/Prehybridization Solution

6 x SSPE
5 x Denhardt's solution
0.5 % SDS

1. Prehybridize membranes in 25 ml of prehybridization solution in a small hybridization tube (for one membrane) or in 30 ml of prehybridization solution in a large hybridization tube (for two membranes), for 1 h at 65 °C.

2. Replace the prehybridization solution with the same volume of pre-warmed hybridization solution.

3. Add the probe and tRNA to the hybridization solution.

4. Place the tube in the rotor and let the hybridization proceed for 16 - 18 h at 65 °C.

2.4.3 - Washing

Washing Solutions

Low Stringency Wash Solution

2 x SSPE
0.1% SDS

High Stringency Wash Solution

0.1 x SSC
O.1% SDS

1. Place 1 - 2 membranes in a baked pyrex dish and wash the membranes in three 200 ml changes of the low stringency wash solution for 20 min each at room temperature on a rotating shaker.

2. Discard these washes in the liquid radioactive waste bottle.

3. Wash the membranes in the same dish in one 200 ml change of the high stringency wash for 5 - 10 min at 65 °C in a shaking water bath.

2.4.4 - Autoradiography and Densitometry

1. Place the membranes in heavy clear plastic bags and seal after removing all of the excess moisture.

2. Place the membranes in x-ray cassettes with two intensifier screens and a piece of X-Omat AR (Kodak) x-ray film expose for 1 - 3 d at -70 °C.

3. Develop the film after 24 h in order to determine the strength of the signal. If the signal is not strong enough for analysis, a new film is placed in the cassette and is exposed for 3 d at -70 °C.

4. Following development of the films, the signals are analysed using an imaging densitometer (BioRad Laboratories, Mississauga, ON) and are expressed in units of adjusted volume (OD x mm^2).

3. Western Blotting

Western blotting is utilized for quantifying protein abundance using antibodies raised against the protein of interest. The proteins are first separated on the basis of size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. The separated proteins are then transferred with electrical current to a membrane. Following this transfer, the membrane is incubated first with an antibody specific to the protein of interest, then is normally incubated with a secondary antibody which is conjugated to a chemiluminescent substance such as horseradish peroxidase. The protein abundance can then be determined using autoradiography followed by densitometry. This procedure is employed in Chapters 2 and 3 of this thesis.

3.1 - Protein Isolation

Solutions for Protein Isolation

Homogenization Buffer:

50 mM Tris-HCl pH 7.5, 0.25 M sucrose, 1 mM EDTA

To make 500 ml:
- 25 ml 1 M Tris-HCl pH 7.5
- 42.8 g sucrose
- 1 ml 0.5 M EDTA pH 8.0

Adjust the volume to 500 ml. Adjust the pH of the solution to 7.5 with HCl.

Pepstatin A

Dissolve 200 mg of pepstatin in 200 ml of ethanol to a final concentration of 1 mg / ml. Store at -20 °C.

Antipain

Dissolve 200 mg of antipain in 200 ml of water to a final concentration of 1 mg / ml. Store at -20 °C.

Leupeptin

Dissolve 2 mg of leupeptin in 200 ml of water to a final concentration of 10 mg / ml. Store at -20 °C.

PMSF

Dissolve 1.74 mg of PMSF in 1 ml of isopropanol to a final concentration of 1.74 mg / ml. Make up fresh each time.

1. Grind 1.0 g of frozen mammary gland tissue in a pre-chilled mortar and pestle on a bed of dry ice. Keep the tissue covered with liquid nitrogen.

2. Transfer the tissue to a 30 ml tube for homogenization.

3. Homogenize the tissue in 3.0 ml of 4 °C homogenization buffer containing 4 µg / ml antipain, pepstatin, leupeptin, 0.5 mM PMSF and 10 mM β-mercaptoethanol, on ice with one 30 s burst with a Polytron power homogenizer.

4. Transfer the homogenate to thick walled polycarbonate tubes (Beckman Instruments Inc., Palo Alto, CA, USA). Keep on ice.

5. Centrifuge the homogenate in a Beckman Type 70.1 ultracentrifuge rotor at 160000 x g (48 000 rpm) for 1 h at 4 °C.

6. Transfer the supernatant to 14 ml Falcon conical tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and freeze at - 70 °C. Discard the pellet.

7. For protein isolation from adipose tissue, centrifuge the supernatant at 15 000 x g (10 000 rpm) for 20 min. Transfer the supernatant to a fresh tube through cheesecloth in order to remove remaining lipid contamination.

3.2 - Quantitation of Protein

1. Quantitate protein using BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA).

2. Prepare BSA standards: 2.0, 1.5, 1.0, 0.75, 0.50, and 0.25 mg/ml.

3. Mix Reagent A with Reagent B in a ratio of 50:1.

4. Dilute sample protein 1:20 with water.

5. Place 10 µl of each standard and samples in the wells of a microtitre plate.

6. Add 200 µl of the reagent mixture to each well.

7. Incubate the microtitre plate at 37 °C for 30 min.

8. Read the plate at 540 nm with a microtitre plate reader.

9. Extrapolate the concentration of protein of the samples from the standard curve.

3.3 - Electrophoretic Separation of Proteins

Solutions for Gel Electrophoresis

1.5 M Tris-HCl, pH 8.8

To 100 ml water add:
27.3 g Tris base

Adjust pH to 8.8 with 10 N HCl. Make the volume up to 150 ml with water.

0.5 M Tris-HCl, pH 6.8

To 60 ml water add:
6 g Tris base

Bring the pH to 6.8 with 10 N HCl. Adjust the volume to 100 ml with water.

30 % Acrylamide

To make 30 ml:

8.76 g acrylamide
0.24 g bisacrylamide

Make up to 30 ml with water and store in cool, dark place for up to 1 week

5 x Loading buffer

To make 8 ml:

50 % glycerol	4 ml glycerol
312.5 mM Tris-HCl, pH 6.8	1.7 ml 1.5 M Tris-HCl, pH 6.8
10 % SDS	0.8 g SDS
25 % β-mercaptoethanol	2 ml β-mercaptoethanol
bromophenol blue	0.2 g bromophenol blue.

Freeze at -20 °C for storage.

5 x Electrode Running Buffer (Stock Solution)

To make 1 L:

15.1 g Tris base
72 g glycine
5 g SDS

Make up to 1 L with water.
* 1 x is the working solution

3.3.1 - Casting the Separating and Stacking Gel

1. Prepare gel casting apparatus for Mini-PROTEAN II gel unit (BioRad Laboratories, Mississauga, ON). Glass plates are prepared by washing them with absolute ethanol. Two 1.5 mm spacers are then placed between the long and the short glass plates and these are clamped into the clamp unit. The glass plates and spacers must be flush at the bottom in order to ensure a tight seal with the rubber gasket of the casting stand. The clamp unit is then placed in the casting stand in preparation for pouring the gel.

2. Before pouring the gel it is necessary to determine that there is a tight seal between the glass plates and the rubber gasket on the casting stand. Flush the glass plates with water to determine if there is any leakage.

3. Prepare the 5 % separating gel.

   5 % separating gel:

   8.4 ml water
   3.8 ml 1.5 M Tris-HCl, pH 8.8
   2.5 ml 30 % acrylamide
   150 µl 10 % SDS
   150 µl 10 % ammonium persulfate **
   12 µl TEMED
   * Add the ammonium persulfate and TEMED just before pouring the gel.
   ** Prepare the ammonium persulfate daily

4. Pour the separating gel between the glass plates to about 1 cm from the top of the smaller plate.

5. Overlay the gel with water saturated-isobutanol.

6. Let the gel polymerize for 45 min then wash the alcohol off the top of the gel with water.

7. Prepare the 4 % stacking gel.

   4 % Stacking Gel:

   6.1 ml water
   2.5 ml 0.5 M Tris-HCl, pH 6.8
   1.3 ml 30 % acrylamide
   100 µl 10 % SDS
   50 µl 10 % ammonium persulfate
   10 µl TEMED

8. Remove all of the water from the top of the separating gel.

9. Pour the stacking gel and place a 15 well 1.5 mm comb into the stacking gel.

10. Add more stacking gel as the gel shrinks around the combs in order to ensure that the volume of the combs is maintained.

11. Let the gel polymerize for 30 min.

12. Following polymerization overlay the gel with water in order to prevent the oxidation of the acrylamide.

3.3.2 - Sample Preparation

1. Dilute samples with homogenization buffer and 5 x loading dye so that the final concentration of protein is 5 mg /ml and the final dye concentration is 1 x.

2. Heat the samples are in a boiling water bath for 10 min to denature the protein.

3. Quench the samples on ice to cool them quickly.

4. Centrifuge the samples momentarily to collect the solution at the bottom of the tube.

5. Rainbow high molecular weight markers (Amersham International plc. Buckinghamshire, England) are prepared by combining 10 μl of the rainbow marker with 2.5 μl of 5 x loading dye and heating the marker in a boiling water bath for 10 min. The samples are then cooled on ice and are centrifuged momentarily to collect the sample.

3.3.3 - Electrophoresis

1. Remove the water from the top of the stacking gel and fill the wells with 1 x electrode running buffer.

2. Place the gels into the holder for the gel unit.

3. Load the volume of sample appropriate to the amount of protein that is required for detection.

4. Place the holder into the gel unit and fill the unit with 1 x electrode running buffer sufficient to cover the electrodes and the bottom of the glass plates.

5. Using a bent pasteur pipette, remove the bubbles from the bottom of the glass plates in order to ensure even voltage across the gel.

6. Fill the reservoir between the two gels with 1 x electrode running buffer.

7. Run the gel for 2 h at 95 V.

3.4 - Transfer of the Proteins to the Membrane

Solutions for Protein Transfer and Antibody Incubation

Towbin's Transfer Buffer pH 8.3

To make 4 L:
12.1 g Tris base
57.6 g glycine
800 ml methanol

Make up to 4 L with water and store at 4 °C.

1. Prepare the NitroPure (Micron Separations Inc., Westborough, MA, USA) membrane, fibre pads and the 3 mm Whatman chromatography paper by soaking them in 4 °C Towbin's buffer.

2. Cut the bottom right corner of the gel before removing them from the glass plates in order to determine the orientation of the gel. Gently remove the gel from the glass plates by slowly moving the glass plate in the Towbin's buffer until the gel slides off the glass plate.

3. Equilibrate the gels for transfer by soaking them in 4 °C Towbin's buffer for 30 min on a shaker.

4. Place a fibre pad on the black surface of the gel holder. Cover with Towbin's buffer then roll the pad with a small glass tube to remove air bubbles.

5. Place a piece of 3 mm Whatman chromatography paper cut to the same size of the gel on the surface of the fibre pad and roll out the air bubbles.

6. Float the gel onto the membrane in the Towbin's buffer. Place the gel face down on the filter paper. Remove the membrane, overlay the gel with Towbin's buffer, and roll out the air bubbles very gently.

7. Place the membrane face down onto the gel. Roll out the air bubbles.

8. Cover the membrane with another piece of filter paper. Roll out the air bubbles.

9. Place the second fibre pad over the filter paper, roll out air bubbles.

10. Close the gel holder so that the grey side of the gel holder is on the top.

11. Place the gel holder in the Mini Trans-Blot (BioRad Laboratories) so that the black side of the gel holder is in contact with the anode and the grey side of the gel holder is in contact with the cathode. The transfer of the proteins from the gel to the membrane is from the negative to positive electrode.

12. Perform the transfer at 250 mA for 2 h or at 40 mA overnight.

13. Following the transfer, confirm the transfer by incubating the membrane in Ponçeau S stain for 5 minutes on a shaker. The membrane is then destained with distilled water. The gel is stained with Coomassie blue by staining for 5 min on a shaker to determine whether there is any protein left on the gel. The gel is the destained with distilled water until the majority of the stain has been removed. If the transfer is poor or incomplete, the membrane will be discarded and the procedure will be repeated.

14. The membranes are stored at 4 °C until they are incubated with the antibody.

3.5 - Antibody Incubation

Solutions for Antibody Incubation

PBS, pH 7.4

To make up 4 L:
32 g NaCl
0.8 g KCl
5.76 g Na_2HPO_4
0.88 g KH_2PO_4

Make up to 4 L with water and adjust pH to 7.4 with concentrated HCl

TBS, pH 7.4

To make 4 L:
32 g NaCl
0.8 g KCl
12 g Tris base

Make up to 4 L with water and adjust pH to 7.4 with concentrated HCl.

TBST

TBS/0.05 % Tween 20

1. **Wash the membranes in two changes of PBS for 5 min each.**

2. **Incubate the membranes in 300 ml of 10 % Blotto / PBS / 0.1 % Tween-20 for 2 h to block non-specific binding.**

3. **Rinse the membrane with TBST.**

4. **Wash in two changes of TBST for 5 min each.**

5. **Incubate the membranes with the primary antibody in the appropriate concentration for 1 h.**

6. Wash the membranes for 15 min in TBST, then in two more changes of TBST for 5 min each.

7. Incubate the membranes with the secondary antibody in the appropriate concentration for 1 h.

8. Wash the membranes with TBST for 15 min, then twice in TBST for 5 min each.

9. Incubate the membranes for 1 min in ECL detection reagent (Amersham International plc) prepared by combining 10 ml of Reagent 1 with 10 ml of Reagent 2.

10. Place the membranes in a heavy plastic bag and remove the excess reagent.

11. Quickly place the membranes in an x-ray cassette with Hyperfilm ECL (Amersham International plc) and expose the film to the membranes for a length of time appropriate to generating a signal for analysis (15 s to 1 min).

12. Develop the film then analyse the results using an imaging densitometer (BioRad Laboratories).